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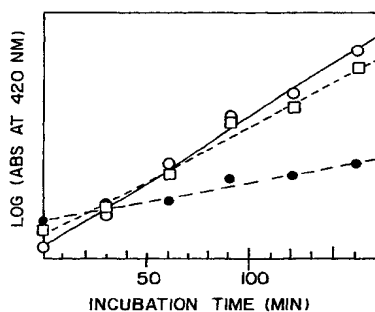
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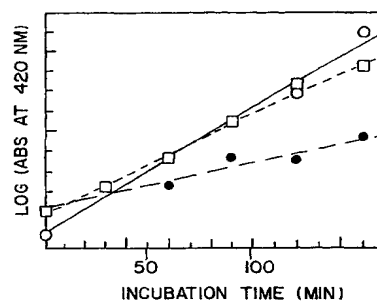
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(54) Title: COMPOSITIONS AND METHODS FOR TARGETED ENZYMATIC RELEASE OF CELL REGULATORY COMPOUNDS



○ CSH23
● CSH23 + 10 µg/ml 5FUR
□ CSH23 + 16.2 µg/ml NSF174



○ CSH22
● CSH22 + 10 µg/ml 5FUR
□ CSH22 + 16.2 µg/ml NSF174

(57) Abstract: Novel pro-drugs and methods for their use to alter the growth and biological characteristics of living cells, tissues, or whole organisms are described. The methods allow for selective activation of the pro-drugs at or near transformant host cells expressing a gene for an enzyme that activates the pro-drugs. Pro-drugs according to a preferred embodiment of the invention are conjugates of a bioactive compound and a chemical group that is capable of being cleaved from the bioactive compound by action of an enzyme. Methods according to this invention include: (a) introducing into targeted cells a gene encoding an enzyme and (b) administering a pro-drug, wherein the enzyme releases the pro-drug from conjugation. In a preferred embodiment of the invention, the gene encoding the enzyme is a marker gene.

COMPOSITIONS AND METHODS FOR TARGETED ENZYMATIC RELEASE OF CELL REGULATORY COMPOUNDS

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9710722 Marker Gene Directed Substrates For Cell Regulation, National Institute of
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Directed Substrates for Cell Regulation.

BACKGROUND OF THE INVENTION

15 I. Field of the Invention.

This invention relates to novel pro-drugs and methods for targeting and
regulating the delivery of the pro-drugs to desired cells and tissues. More particularly, the
invention relates to the use of transferred genes, preferably reporter or marker genes, with
matched pro-drugs that are activated only in the transformed cells and the use of such
20 systems for selecting transformed cells, affecting the cell growth or characteristics of the
transformed cells, or as a means of drug targeting to specific cells or tissues in a mixed
cell population.

II. Description of the Prior Art.

Targeted and Regulated Drug Delivery. Targeted and regulated delivery
25 of biochemical agents has been investigated for a variety of uses. For example, targeted
drug delivery systems have the potential to provide a mechanism for delivering cytotoxic
agents directly to cancerous cells, tissue specific drugs to the lung epithelium for cystic
fibrosis treatment, or analgesic drugs for chronic (neuropathic) pain treatment. In cancer
treatment, the selective delivery of cytotoxic agents to tumor cells is desirable because
30 systemic administration of these agents often kills normal cells within the body as well as
the tumor cells sought to be eliminated. Antitumor drug delivery systems currently in use

typically utilize a cytotoxic agent conjugated to a tumor-specific antibody to form an immunoconjugate. This immunoconjugate binds to tumor cells and thereby "delivers" the cytotoxic agent to the site of the tumor. Despite the amount of research directed toward the use of immunoconjugates for therapeutic purposes, several limitations involved in these delivery approaches have become apparent (see, e.g., Embleton, Biochem. Society Transactions, 14: 393, 615th Meeting, Belfast, 1986). For example, the large amount of drug required to be delivered to the target tumor cell to effect killing of the cell is often unattainable because of limitations imposed by the number of tumor-associated antigens on the surface of the cells and the number of drug molecules that can be attached to any given antibody molecule.

Methods of cloning genes for bioactive compounds directly into the cells to be affected also have been attempted. The use of cloned gene expression for cell regulatory purposes typically involves effecting cell ablation using a gene expression event. Attempts to express a toxic gene product in cells have not been generally successful. Toxic gene products are difficult to regulate. In addition, cells expressing a toxic product often down-regulate the expression of this toxic product in order to survive. Selection for cells that can survive despite the cloned gene activity also occurs. For example, the diphtheria toxin A-subunit has been transcribed under regulation of a developmentally regulated promoter system in tobacco plants for specific tissue ablation (Koltunow et al., 1990). Mariani et al. (1990) described expression of a chimeric ribonuclease gene to destroy reproductive cells in plants. Recent reports have described selective release strategies for naturally occurring, inactive phytohormone glycoside conjugates (β -glucosides) in cloned plant tissues using a *Zea mays* cDNA in tobacco roots (Brzobohaty, B., et al, 1993). Gene therapy techniques that include use of a chimeric gene encoding a fusion protein capable of increasing activity of pyrimidine analogs (Tiraby, R., et al., 1996) or combined suicide-gene techniques (Chen, S.H., et al., 1996) have also been reported.

Marker Genes. Studies in the genetic and molecular basis of eukaryotic growth and differentiation have led to advances in many important areas, including the control of the cell cycle during development, the mechanism of the maternal effect on embryogenesis, and the molecular genetic basis of pattern formation. A byproduct of

these studies has been the development of “reporter” or “marker” genes that are used as tools in such work. Studies with a variety of organisms and experimental systems have revealed that many important aspects of eukaryotic development are controlled by the differential expression of genetic information. Tissue specific promoters involved in the expression of developmentally important genes have been identified in various species. Marker genes are commonly used to monitor the effectiveness of these promoters.

Marker genes have been used outside of the study of tissue specific promoters. Expression of foreign genes in mammalian and plant cells has become the quintessential biotechnology protocol, and co-expressed marker genes have been used for several decades to track the expression levels of the simultaneous cloned genes (Roederer, M., et al., 1991). The activity encoded by a chimeric gene construct introduced by techniques of genetic engineering is sensitive, even to the point of measuring activity in single cells (Naleway, 1992; Naleway, et al., 1991). It has become conventional to construct and study gene fusions in which marker gene activity is restricted to particular cell types, tissues, organs, or stages of development.

Marker genes also have been used in combination with a suitable substrate to provide for detection of the protein expression. The substrate is applied to transgenic cells containing an active reporter or marker gene coding for an enzyme, the substrate is enzymatically turned over to a product that can be easily detected by visual or spectrophotometric techniques. Examples of such systems include the use of chromogenic substrates (like 5-bromo-4-chloroindolyl galactoside (X-Gal) for detection of *lacZ* β -galactosidase activity in cells and tissues (Lim, K., *et al.*, 1989; Marsh, J., 1994)), fluorogenic substrates (like 4-methylumbelliferyl glucuronide (MUG) for detection of β -glucuronidase activity in plant cells or tissues (Jefferson, R.A., 1988)), or bioluminescent substrates (like luciferin for detection of cloned firefly luciferase activity in various cell or tissue types (Wood, K.V., *et al.*, 1989)). In a related technique, a selection marker (gene) may also be used to confer antibiotic resistance to cells, tissues or organisms when a matched antibiotic is applied to the transgenic cells.

Certain qualities are necessary in a useful marker gene: typically, the lack of detectable intrinsic enzyme activity in the recombinant cells, the robust nature of the marker (usually a bacterial enzyme) and the availability of substrates to estimate the

enzyme cloned activity allow sensitive detection of the marker gene (and therefore of any co-expressed gene of interest). The marker genes that are available for use during the genetic engineering of plants or animal cells have found widespread use in molecular biology and biotechnology for the selection, detection and analysis of transgenic cells or tissues.

E. coli lacZ Marker Gene. The most frequently used reporter gene is probably the *Escherichia coli lacZ* gene, which encodes an active subunit of β -galactosidase (Lis, et al., 1984 and Beckwith, et al., 1970). The bacterial *lacZ* β -Galactosidase enzymatic activity can be easily and sensitively measured, it can be expressed and assayed in virtually any type of cell, and its activity is unaltered by making N-terminal fusion polypeptides.

Firefly luciferase (luc) Marker Gene. The *luc* gene from the American firefly *Photinus pyralis* (de Wet, et al., 1985, de Wet, et al., 1987, Brasier and Ron, 1992) has been widely used as a reporter of cloned gene activities in both plant and animal cells and tissues, both *in vivo* and *in vitro* using an assay with added ATP and appropriate buffer (containing Mg^{+2}). This assay is extremely sensitive, allowing detection of subattomole concentrations of enzyme.

Amp Selection Marker. The selection markers for antibiotic resistance (i.e. *amp* for ampicillin resistance and *tet* for tetracycline resistance) are also some of the most widely used marker genes, now routinely incorporated into bacterial plasmid vectors (Bolivar, et al., 1977a; Bolivar, et al., 1977b). Since the (second) tetracycline resistance gene of many plasmids (e.g. pBR322) often contains the cloning site, this leaves ampicillin resistance as a major method of screening recombinant cells in many industrial biotechnology systems. Recombinant cells become capable of detoxifying the antibiotic ampicillin (applied to the media at a concentration of about 50 μ g/mL), and are “selected”, while non-transformed cells are ablated.

Other Marker Genes. A list of common marker genes with their detection methods is given below:

Reporter gene	Detection method (reagent)
Acid phosphatase	Colorimetric

Aequorin (<i>phot</i>)	Bioluminescent (coelenterazine) Colorimetric
Alcohol dehydrogenase	Colorimetric
Alkaline phosphatase	BL (luciferin phosphate) Chemiluminescent (CSPD) Colorimetric (PNPP) Colorimetric (BCIP) Colorimetric (AS-MXP)
Aminoglycoside phosphotransferase (<i>aph</i> (3') Catechol 2,3-dioxygenase (<i>xylE</i>) Chloramphenicolacetyltransferase (CAT)	Autoradiography ([¹⁴ C]chloramphenicol) Fluorescence (Bodipy chloramphenicol) Scintillation Counting ([³ H]acetyl-CoA) Immunoassay
Firefly luciferase (<i>luc</i>)	Bioluminescence (Firefly luciferin-ATP)
Galactokinase	
β-Galactosidase (<i>lac Z</i>)	Bioluminescence Colorimetric (ONPG) Colorimetric (X-GAL) Fluorescence (FDG) Fluorescence (MUGal)
β-Glucuronidase (<i>gusA</i> , <i>uidA</i>)	Chemiluminescence Colorimetric (X-GlcU) Fluorescence (MUGlcU)
Growth hormone	Immunoassay
Interleukin-2 (IL-2)	
Marine bacterial luciferase (<i>lux A/B</i>)	Bioluminescence (FMNH ₂ -decanal)
Neomycin phosphotransferase (<i>neo</i>)	
Ornithine transcarbamylase	
Phosphinothricin acetyltransferase (<i>bar</i>)	
Puromycin acetyltransferase (<i>pac</i>)	
Renilla luciferase (<i>luc</i>)	Bioluminescence

Thaumatococcus	
Thymidine kinase	
Xanthine-guanine phosphoribosyltransferase	
<i>Vargula</i> luciferase	

Note: CSPD, disodium 3-(4-methoxy spiro[1,2-dioxetane- 3,2'-(5'-chloro)-tricyclo[3.3.1.1]decan]-4-yl phenyl phosphate; FDG, fluorescein digalactoside; X-GlcU, 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid; MUGal, 4-methylumbelliferyl β -D-galactopyranoside; MUGlcU, 4-methylumbelliferyl β -D-glucuronide; NET, nitro blue tetrazolium; ONPG, O-nitrophenyl β -D-galactopyranoside; PNPP, 4-nitrophenyl phosphate; X-GAL, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside.

SUMMARY OF THE INVENTION

The present invention provides methods and compounds to permit selection, detection or analysis of plant and animal cells during genetic engineering or to influence the growth or biological characteristics of cells or the organisms that contain these cells. It is an object of the present invention to extend the use of active marker genes beyond their use for detection of gene activity in living cells. A primary object of the invention is to allow targeting of novel pro-drugs to transgenic cells that contain a gene for an enzyme, preferably an active marker gene. The pro-drugs also may be targeted to surrounding cells or tissues. The present invention makes it possible to introduce an inactive biological agent into selected cells in culture, and thereby into selected tissues or whole organisms where it becomes activated by use of a cloned gene activity. Since the cloned gene for the enzyme is to be complementary to the pro-drug, release of the activated bioactive compound from the pro-drug form can be restricted to only those cells having the cloned gene in a mixed cell population or in tissues or whole organisms. Molecular biology techniques, such as the use of activatable promoter elements, cell or tissue specific promoters, or other cis- and trans-acting factors, capable of controlling gene expression may further restrict activation of the pro-drugs dependent upon expression of the cloned gene. The present invention provides compositions and methods for drug targeting and treatment for a variety of diseases including certain forms

of cancer, cystic fibrosis, autoimmune diseases, proliferative and degenerative diseases, as well compositions and methods for use in agricultural biotechnology, the production of new proteins and drugs in cell-culture systems, bacterial screening strategies, and non-destructive selection mechanisms.

5 Other objects, advantages and features of the invention will be apparent from the following descriptions.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows growth plots of two *E. coli* strains, CSH23/S90C and CSH22/S90C, grown with 5-fluorouridine (5FUR) and 5-fluorouridine-5'-O- β -D-galactopyranoside (M0174).

Fig. 2 shows growth plots of two *E. coli* strains, W3110 and CSH36 grown in the presence of ONPG. Duplicate trials (A and B) were run. W3110 was grown with (W3110i) and without (W3110) the addition of INTG.

Fig 3 shows western blots of lacY and lacZ expression in D5 clones. A. pYDisplay clone A2. AntiHA probing shows a unique doublet band of unexpected size present in both membrane (M) and cytosolic (C) fractions. B. pZDisplay clones B7 and B12. Anti- β -galactosidase probing shows bands corresponding to the same size as standard β -gal protein (S) and β -gal expressed in D5A2 cells. β -gal in D5A2 cells is present primarily in cytosol fractions, while B7 clone seemed to have more present in membrane fractions.

DETAILED DESCRIPTION

I. Definitions.

The term "drug" as used in this application refers to any bioactive substance that has a positive or negative effect on cell growth, morphology, gene expression, immunochemistry, or biology.

The term "pro-drug" as used in this application refers to a precursor or derivative form of a bioactive compound that is less active compared to the parent bioactive compound and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, 1986 and Stella et al., 1985.

The term "biological activity" or "bioactive compound" is used to refer to any activity or a compound having an activity that can be assayed or measured including,

but not limited to, any binding, blocking, chelating, enzymatic, agonistic, antagonistic, inhibitory, stimulatory, metabolic or structural activity.

II. Preferred Embodiments.

According to one preferred embodiment of the present invention, the
5 growth or biological characteristics of a genetically transformed mammalian, plant or bacterial cell sample are influenced by introducing a foreign gene or gene portion expressing the *lacZ* β -galactosidase enzyme; exposing the transgenic cells to a specific β -galactoside conjugate, and culturing the cells either *in vitro* or *in vivo*. Selection of the transgenic mammalian, plant or bacterial cell can be effected by utilizing a β -galactoside
10 conjugate that improves the growth or viability of this cell at the expense of non-transformed cells, by application of a β -galactoside conjugate that releases an essential vitamin, growth factor, nutrient, hormone, phytohormone, or the like, or that satisfies an artificial or externally imposed block in metabolism.

According to another preferred embodiment of the present invention, the
15 growth or biological characteristics of a genetically transformed mammalian, plant or bacterial cell sample are influenced by introducing a foreign gene or gene portion expressing the *E. coli* β -glucuronidase gene in the cells; exposing the transgenic cells to a specific β -glucuronide conjugate, and culturing the cells either *in vitro* or *in vivo*. Selective release of the conjugate is affected in a tissue or organ specific manner is
20 achieved by use of tissue specific promoter control, or by inducing expression of the foreign gene in response to an externally applied signal by the use of an environmentally sensitive promoter system (circadian rhythm, heat-shock, inducible or other like promoter system).

According to yet another preferred embodiment of the present invention,
25 the growth or biological characteristics of a genetically transformed mammalian, plant or bacterial cell sample are influenced by introducing a foreign gene or gene portion expressing the firefly luciferase gene (Photinus-luciferin:oxygen 4-oxireductase [decarboxylating, ATP-hydrolysing] (EC 1.13.12.7)), into either plant, bacterial or animal cells; exposing the transgenic cells to a specific D-luciferin conjugate of a bioactive
30 compound, and culturing the cells either *in vitro* or *in vivo*, thereby influencing the growth

or biological characteristics of the genetically transformed mammalian, plant or bacterial cell sample.

According to another preferred embodiment of the present invention, the growth or biological characteristics of a genetically transformed mammalian, plant or bacterial cell sample are influenced by introducing a foreign gene or gene portion
5 expressing the ampicillin resistance gene (ampicilinase, *amp*, β -lactamase) wherein a plant, animal or bacterial cell sample is contacted with a specific cephalosporin-type conjugate of a bioactive compound, and cultured or *in vivo* growth maintained, thereby influencing the growth or biological characteristics of the genetically transformed
10 mammalian, plant or bacterial cell sample containing a foreign gene or gene portion expressing the β -lactamase enzyme.

In another preferred embodiment of the invention, the activated drug product is translocated to neighboring cells or tissues, by enzymatic release within the transgenic cells, when the pro-drug is applied to a mixed cell population containing both
15 transgenic and non-transgenic cells. The effect of the active product on neighboring non-transgenic cells (so-called "by-stander effect") elicits drug targeting in these tissues and within whole organisms.

Other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

20 **A. Pro-drugs and Matched Enzymes.**

Many marker genes may be used in the present invention. The marker gene will code for an enzyme activity that is atypical of the cells or tissues being employed. Any pro-drug that is activated by the enzyme expressed in the transformed cells may be used in the present invention. The variety of pro-drugs that may be
25 produced and used in the present invention include, but are not limited to, members of the following classes of bioactive compounds: immuno-stimulatory compounds including Phorbol Esters (Phorbol Myristate Acetate), Lipid A analogs and diacylglycerols, Retinoic Acid, insulin, cyclic nucleotides (cAMP), and protein kinase C activators as well as nutrients (serum components such as essential amino acids), D-glucose (lactose),
30 hormones (including dexamethasone, dihydrotestosterone, aldosterone, progesterone, other glucocorticoid hormones, L-thyroxine and triiodo-L-thyronine), vitamins (including

choline, folic acid, myo-inositol, niacinamide, pyridoxal, riboflavin, pantothenic acid, thiamine) toxins (for example, cycloheximide and psoralen), enzyme inhibitors (preferably specific kinase or glycosidase inhibitors), plant herbicides such as bromoxynil (4-hydroxy-3,5-dibromobenzonitrile), atrazine (2-chloro-4-ethylamino-6-isopropylamino-
5 *sym*-triazine), dinoseb (2-*sec*-butyl-4,6-dinitrophenol), phosphinothricin, 2'-deoxyadenosine, 3'-deoxyadenosine (cordycepin) and ara-A, angiogenesis inhibitors (for example, endostatin and angiostatin), antitumor chemotherapeutic compounds (for example, doxorubicin, mitoxantrone, 5-fluorouridine and 5-fluorouracil, resveratrol, methotrexate, or 4-hydroxyphenylretinamide), as well as all classes of analgesic and
10 therapeutic drugs that are known to exhibit positive or negative effects on the growth and development of cells. The factors that contribute to making a drug reliable and easy to conjugate are the availability of an aliphatic or aromatic hydroxyl, amino, thio, carboxyl or aldehyde group that is located at a position in the drug or bioactive compound whereby conjugation at this position reduces or eliminates biological activity.

15 The preferred substrates of this invention are drug conjugates represented by the general formula:



wherein the group $\text{DRUG}_{(-\text{XH})}$ represents a portion of a bioactive compound as further described below, known to have a strong positive or negative effect
20 on the biological function, growth or characteristics of a cell, tissue or organism. The native bioactive compound generally has the formula $\text{H-X-DRUG}_{(-\text{XH})}$ in its active form. The bioactive compound maybe for example, an antimetabolite, enzyme inhibitor, essential vitamin, essential nutrient, growth regulatory compound, a compound capable of inducing gene expression, hormone, or cytokinin.

25 BLOCK represents a group that changes the biological activity of the bioactive compound, and is capable of being cleaved from the substrate molecule by action of an enzyme. Preferably BLOCK causes a significant reduction or elimination of activity for the bioactive compound in the substrate. BLOCK is selected to be specific for the enzyme of interest. Preferably BLOCK is a monovalent moiety derived by removal of
30 a hydroxyl group from an alcohol or from a carboxy group of an aliphatic, aromatic or amino acid, or of a peptide; or a monovalent moiety derived by removal of the anomeric

hydroxyl group from a mono- or polysaccharide. Some preferred monovalent blocking groups and the enzymes that will cleave such groups from the substrate are listed below. Additional BLOCK groups, or derivatives or analogs of the described BLOCK groups listed below also may be used in the present invention.

5

E.C. Number	ENZYME	BLOCK Group
3.2.1.1	α -amylase	(1-4) α D-Glucose
3.2.1.2	β -amylase.	(1-4) β D-Glucose
3.2.1.3	Glucan 1,4- α -glucosidase.	(1-4) α D-Glucose
3.2.1.4	Cellulase.	(1-4) β D-Glucose
3.2.1.7	Inulinase.	(2-1) β D-Fructose
3.2.1.11	Dextranase	(1-4) β D,(1-3) α D-Glucose
3.2.1.14	Chitinase.	(1-4) β D-GlcN
3.2.1.15	Polygalacturonase	(1-4) α D-GlcU
3.2.1.18	Exo- α -sialidase.	N-Ac-Neuraminic Acid
3.2.1.20	α -Glucosidase	α -D-Glucose
3.2.1.21	β -Glucosidase	β -D-Glucose
3.2.1.22	α -Galactosidase	α -D-Galactose
3.2.1.23	β -Galactosidase	β -D-Galactose
3.2.1.24	α -Mannosidase	α -D-Mannose
3.2.1.25	β -Mannosidase	β -D-Mannose
3.2.1.30	N-Acetyl- β - Glucosaminidase	β -D-N-Acetylglucosamine
3.2.1.31	β -Glucuronidase	β -D-Glucuronic acid
3.2.1.37	Xylan 1,4- β -xylosidase	1,4- β -xylose
3.2.1.38	β -D-fucosidase	β -D-Fucose
3.2.1.40	α -L-rhamnosidase	α -L-Rhamnose
3.2.1.43	β -L-rhamnosidase	β -L-Rhamnose
3.2.1.49	α -N- acetylgalactosaminidase	α -N-Ac D-Galactose

3.2.1.50	α -N-acetylglucosaminidase	α -N-Ac D-Glucose
3.2.1.51	α -L-fucosidase	α -L-Fucose
3.2.1.52	β -N-acetylhexosaminidase	β -N-Ac D-Glucose
3.2.1.53	β -N-acetylgalactosaminidase	β -N-Ac D-Galactose
3.2.1.55	α -L-arabinofuranosidase	α -L-Arabinofuranose
3.2.1.76	L-iduronidase	L-Iduronic acid
3.2.1.80	Fructan β -fructosidase	β -Fructose
3.2.1.88	β -L-arabinosidase	β -L-Arabinose
3.2.1.108	Lactase	Lactose
3.2.1.112	2-deoxyglucosidase	2-Deoxyglucose
3.2.3.1	Thioglucosidase	1-Deoxy-1-thio-Glucose
3.5.2.6	β -lactamase	Penicillin, Cephalosporin
1.13.12.5	Renilla-luciferin	2-monooxygenase Aequorin
1.13.12.6	Cypridina-luciferin	2-monooxygenase
1.13.12.7	Photinus-luciferin (4-4-monooxygenase); Firefly luciferase; Luciferase	D-Luciferin (5-Hydroxymethyl-D-Luciferin)
1.13.12.8	Watasemia-luciferin	2-monooxygenase

The group H-X represents an atom or atoms in the structure of the native bioactive compound where attachment of a BLOCK group is facilitated. The group H-X can include, but is not limited to, hydroxyl (-OH), amino (-NH₂), thiol (-SH), carboxyl (-COOH), aldehyde (-CHO), carbamyl (-CONH₂), or any other group that allows stable, specific, enzyme cleavable attachment of a BLOCK group to the bioactive compound. The group X represents the remaining atoms of H-X after removal of a hydrogen (H) atom by chemical attachment.

The position of the group H-X in the native drug compound is chosen so that the resulting substrate molecule can, by action of an enzyme, release the native drug

in its active form, and so that attachment of the BLOCK group reduces or eliminates such activity.

The group L represents a linking group that may or may not be present in the substrate. This linking group may be present to facilitate chemical synthesis of the final substrate. Preferably L is a divalent moiety linking BLOCK with X. The group L must itself be either enzyme cleavable by a ubiquitous enzyme activity, or become unstable and be removed under physiological conditions after enzyme activity releases the BLOCK group. The group L can include, but is not limited to the carbonyl (C=O) group, as part of a carbonate, carbamate thiocarbonate, or other linkage in the final substrate.

The pro-drugs are formed in general as follows: For glycosides, the (suitably protected) bioactive compound is reacted under anhydrous conditions with acetobromogalactose, acetochloroglucuronic acid, methyl ester, or other activated sugar, using a silver or mercury type catalyst (silver carbonate, silver triflate, silver oxide and iodine, etc.) and non-nucleophilic base (sym-collidine) in an anhydrous solvent (like dichloromethane) for several days at room temperature in the dark. After purification of the fully protected conjugate by standard silicagel chromatography conditions, the protected conjugate is deprotected using catalytic anhydrous base (such as sodium methoxide, lithium hydroxide, etc.). For cephalosporin derivatives, a suitably protected cephalothin derivative such as 7-N-benzyl-cephalosporanic acid, diphenylmethylester is esterified with a bioactive compound (or protected bioactive compound), using standard techniques (dicyclohexylcarbodiimide, catalytic dimethylaminopyridine in dichloromethane). After purification using silicagel chromatography techniques, the completely protected conjugate is deprotected using trifluoroacetic acid, and/or other agents for the drug deprotection. For D-luciferin derivatives, 5-Hydroxymethyl-D-Luciferin is reacted with one equivalent of dicarbonylimidazole and the bioactive compound or suitably protected bioactive compound analog added (1 equivalent). This product, after purification as above, is deprotected and purified to homogeneity as above.

Some examples of pro-drugs and specific methods of forming the them are described below and in the Examples. The number, classes and types of drugs or bioactive compounds is, however, not limited to these examples:

- Cycloheximide 2-O- β -D-Galactopyranoside (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-O- β -D-galactopyranosyl]ethyl]glutarimide),
- Dexamethasone 21-O- β -D-galactopyranoside (21-O- β -D-galactopyranosyl-9-fluoro-11,17- dihydroxy-16-methylpregna-1,4-diene-3,20-dione,
- 5 Dexamethasone 11-O- β -D-galactopyranoside (21-O- β -D-galactopyranosyl-9-fluoro-21,17- dihydroxy-16-methylpregna-1,4-diene-3,20-dione,
- Dexamethasone 17-O- β -D-galactopyranoside (21-O- β -D-galactopyranosyl-9-fluoro-21,11- dihydroxy-16-methylpregna-1,4-diene-3,20-dione,
- Dexamethasone 21,11-di-O- β -D-galactopyranoside (21-O- β -D-galactopyranosyl-11-O- β -D-galactopyranosyl -9-fluoro-17-hydroxy-16-methylpregna-1,4-diene-3,20-dione,
- 10 Dexamethasone 21,17-di-O- β -D-galactopyranoside (21-O- β -D-galactopyranosyl-17-O- β -D-galactopyranosyl -9-fluoro-11-hydroxy-16-methylpregna-1,4-diene-3,20-dione,
- Dexamethasone 11,17-di- β -O-D-galactopyranoside (11-O- β -D-galactopyranosyl-17-O- β -D-galactopyranosyl -9-fluoro-21-hydroxy-16-methylpregna-1,4-diene-3,20-dione,
- 15 Dexamethasone 11,17,21-tri- β -O-D-galactopyranoside (11-O- β -D-galactopyranosyl-17-O- β -D-galactopyranosyl -11-O- β -D-galactopyranosyl-9-fluoro-16-methylpregna-1,4-diene-3,20-dione,
- 4'-O- β -D-galactopyranosylmethyl-2,5,9-trimethylpsoralen,
- Chloramphenicol 3-O- β -D-galactopyranoside (D(-)-threo-2,2-dichloro-N-[β -hydroxy- α -
- 20 (O- β -D-galactopyranosylmethyl)- β -(4-nitrophenyl)ethyl]acetamide,
- 5-Fluorouridine-5'-O- β -D-Galactopyranoside,
- Tetracycline 10-O- β -D-Galactopyranoside [(4S-(4a,4aa,5aa,6b,12aa))-4-(dimethylamino)-1,4,4a,5,5a,6-11,12a-octahydro-3,6,12,12a-tetrahydroxy-10-O- β -D-galactopyranosyl-6-methyl-1,11-dioxo-2-naphthalenecarboxamide),
- 25 Tetracycline 3-O- β -D-Galactopyranoside [(4S-(4a,4aa,5aa,6b,12aa))-4-(dimethylamino)-1,4,4a,5,5a,6-11,12a-octahydro-6,10,12,12a-tetrahydroxy-10-O- β -D-galactopyranosyl-6-methyl-1,11-dioxo-2-naphthalenecarboxamide),
- Tetracycline 6-O- β -D-Galactopyranoside [(4S-(4a,4aa,5aa,6b,12aa))-4-(dimethylamino)-1,4,4a,5,5a,6-11,12a-octahydro-3,10,12,12a-tetrahydroxy-10-O- β -D-galactopyranosyl-6-methyl-1,11-dioxo-2-naphthalenecarboxamide),
- 30

- Tetracycline 12-O-β-D-Galactopyranoside [(4S-(4a,4aa,5aa,6b,12aa)]-4-(dimethylamino)-1,4,4a,5,5a,6-11,12a-octahydro-3,6,10,12a-tetrahydroxy-10-O-β-D-galactopyranosyl-6-methyl-1,11-dioxo-2-naphthalenecarboxamide),
- Tetracycline 12a-O-β-D-Galactopyranoside [(4S-(4a,4aa,5aa,6b,12aa)]-4-(dimethylamino)-1,4,4a,5,5a,6-11,12a-octahydro-3,6,10,12-tetrahydroxy-10-O-β-D-galactopyranosyl-6-methyl-1,11-dioxo-2-naphthalenecarboxamide),
- 5 Dexamethasone 21-O-β-D-galactopyranoside (21-O-β-D-galactopyranosyl-9-fluoro-11,17- dihydroxy-16-methylpregna-1,4-diene-3,20-dione),
- Dexamethasone 11-O-β-D-galactopyranoside (11-O-β-D-galactopyranosyl-9-fluoro-17,21- dihydroxy-16-methylpregna-1,4-diene-3,20-dione),
- 10 Dexamethasone 17-O-β-D-galactopyranoside (17-O-β-D-galactopyranosyl-9-fluoro-11,21- dihydroxy-16-methylpregna-1,4-diene-3,20-dione),
- Dexamethasone 11,21-di-O-β-D-galactopyranoside (11,21-di-O-β-D-galactopyranosyl-9-fluoro-17-hydroxy-16-methylpregna-1,4-diene-3,20-dione),
- 15 Dexamethasone 11,17,21-tri-O-β-D-galactopyranoside (11,17,21-tri-O-β-D-galactopyranosyl-9-fluoro-16-methylpregna-1,4-diene-3,20-dione),
- Dexamethasone 17,21-di-O-β-D-galactopyranoside (11,21-di-O-β-D-galactopyranosyl-9-fluoro-11-hydroxy-16-methylpregna-1,4-diene-3,20-dione),
- Dexamethasone 11.17-di-O-β-D-galactopyranoside (11,21-di-O-β-D-galactopyranosyl-9-fluoro-21-hydroxy-16-methylpregna-1,4-diene-3,20-dione),
- 20 N-Benzyl Cephalothin L-glutamate (6R[6a,7b(R*)]-3-a-(aminoglutyryloxy)methyl)-7-(phenylacetyl)amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid),
- Doxorubicin 4'-O-β-D-galactopyranoside (8S-cis)-10-[3'-amino-4'-O-β-D-galactopyranosyl-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- 25 Doxorubicin 6-O-β-D-galactopyranoside (8S-cis)-10-[3'-amino-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-8,11-dihydroxy-8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- 30

- Doxorubicin 8-O- β -D-galactopyranoside (8S-cis)-10-[3'-amino-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- 5 Doxorubicin 11-O- β -D-galactopyranoside (8S-cis)-10-[3'-amino-4'-O- β -D-galactopyranosyl-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8-dihydroxy-8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- Doxorubicin 4',6,8-tri-O- β -D-galactopyranoside (8S-cis)-10-[3'-amino-4'-O- β -D-galactopyranosyl-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8-di-O- β -D-galactopyranosyl 11-hydroxy-8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- 10 Doxorubicin 4',6,8,11-tetra-O- β -D-galactopyranoside (8S-cis)-10-[3'-amino-4'-O- β -D-galactopyranosyl-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-tri-O- β -D-galactopyranosyl -8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- 15 Doxorubicin 4',6-di-O- β -D-galactopyranoside (8S-cis)-10-[3'-amino-4'-O- β -D-galactopyranosyl-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6-O- β -D-galactopyranosyl-8,11-dihydroxy-8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- Doxorubicin 4',8-di-O- β -D-galactopyranoside (8S-cis)-10-[3'-amino-4'-O- β -D-galactopyranosyl-2',3',6'-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-8-O- β -D-galactopyranosyl-6,11-dihydroxy-8-hydroxyacetyl)-1-methoxy-5,12-naphthacenedione),
- 20 Resveratrol 4'-O- β -D-galactopyranoside, Resveratrol 3-O- β -D-galactopyranoside, Resveratrol 5-O- β -D-galactopyranoside, Resveratrol 4',3-di-O- β -D-galactopyranoside,
- 25 Resveratrol 5,3-di-O- β -D-galactopyranoside, Resveratrol 5,4',3-tri-O- β -D-galactopyranoside,
- Phorbol 12-O-octanoyl-13-O-acetyl- 6b-O- β -D-galactopyranoside,
- Phorbol 12-O-octanoyl-13-O-acetyl-4-O- β -D-galactopyranoside,
- Phorbol 12-O-octanoyl-13-O-acetyl-10-O- β -D-galactopyranoside,
- 30 Phorbol 12-O-octanoyl-13-O-acetyl-4,6b-O-di- β -D-galactopyranoside,
- Phorbol 12-O-octanoyl-13-O-acetyl-4,10-di-O- β -D-galactopyranoside,

- Phorbol 12-O-octanoyl-13-O-acetyl-6b,10-di-O- β -D-galactopyranoside,
 Phorbol 12-O-octanoyl-13-O-acetyl-4,6b,10-tri-O- β -D-galactopyranoside,
 1,2-di-O-octanoyl-3-O- β -D-galactopyranosyl-*rac*-glycerol,
 4-O-(β -D-galactopyranosyl)phenyl retinamide,
 5 Mitoxantrone 2',2'-bis-di-O- β -D-galactopyranoside (1,4-dihydroxy-5,8-bis[2(2-O- β -D-galactopyranosylethyl)aminoethylamino]-9,10-anthracenedione),
 Thymidine 5'-O-- β -D-galactopyranoside (1-(2'-deoxy-5'-O- β -D-galactopyranosyl-(β -D-ribofuranosyl)-5-methyluracil),
 2'-O-(2,6-dibromo-4-cyano-phenyl)oxymethyl) D-Luciferin,
 10 myo-inositol β -D-galactoside,
 pantothenic acid di- β -D-galactoside,
 pyridoxal 5-O- β -D-galactoside, and
 pyridoxine β -D-galactoside.
 2',3'-dideoxy-2',3'-didehydroadenosine 5'-O-galactopyranoside
 15 (d4A-Gal)
 tryptophan-cephalosporanate
 4-O-Methotrexate b-D-galactopyranoside
 RGDS-Galactoside (Arginine-Glycine-Aspartic
 Acid-Serine-O-b-D-Galactopyranoside)
 20 Swainsonine b-D-Galactopyranoside
 beta-oestradiol 2,17-di-O-b-D-galactopyranoside
 12-Crown-4 2-O-b-D-galactopyranoside

B. Transfection of Cells

- 25 In the present invention, cells are transformed or transfected either *in vivo* or *ex vivo* with a recombinant expression vector capable of transforming the cells to express an enzyme such as those described in paragraph II.A., above. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the
 30 invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a

cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, 5 DEAE-dextran-mediated transfection, lipofection, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al.1989, and other laboratory textbooks.

The nucleic acids of the present invention which encode enzymes having the appropriate activity as described in paragraph II.A., above, can be incorporated in a 10 known manner into a recombinant expression vector to obtain the desired expression of the encoded enzyme. The recombinant expression vectors contain a nucleic acid or an oligonucleotide fragment thereof of the invention and a regulatory sequence, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid or oligonucleotide fragment. Operatively linked is intended to mean that the 15 nucleic acid is linked to a regulatory sequence in a manner which allows expression of the nucleic acid. Regulatory sequences are art-recognized and are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the regulatory sequence may be a promoter, enhancer or other expression control elements. Such regulatory sequences are known to those skilled in the art or one described in Goeddel, 20 1990, can be used. The expression vector may contain activatable promoter elements, inducible promoter systems, tissue or environment sensitive promoter systems, and-or other cis- or trans-acting factors effecting gene expression *in vivo*. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transfected and/or the type of enzyme desired to be expressed.

25 The recombinant expression vectors of the invention can be designed for expression of encoded proteins in prokaryotic or eukaryotic cells. For example, proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, 1990. Expression in prokaryotes is most often carried out in *E. coli* with vectors containing 30 constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1

(Baldari. et al., 1987), pMFa (Kurjan and Herskowitz, 1982), pjRY88 (Schultz et al., 1987), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., 1983) and the pVL series (Lucklow, V. A., and Summers, M. D.,
5 1989).

C. Applications.

The pro-drugs of the present invention may be used to treat a variety of diseases, such as cancer, cystic fibrosis, chronic bacterial infection, viral infection, Duchenne's Muscular Dystrophy, arthritis, coronary artery disease, chronic pain or other
10 genetic, autoimmune, proliferative or degenerative diseases. The pro-drugs also may be used in positive selection mechanisms.

Various cancers, such as human melanoma, human ovarian cancer, human neuroblastoma, human squamous cell carcinoma, human fibrosarcoma, human prostate cancer, and human pancreatic cancer, may be treated. For treating cancer, the pro-drug is
15 a cytotoxic compound, such as doxorubicin, mitoxantrone, 5-fluorouridine, 5-fluorouracil, methotrexate, 4-hydroxyphenylretinamide, cyclohexamide, or cyclophosphamide, conjugated with a BLOCK. Preferably, a vector or retroviral vector that contains an enzyme selected to match the pro-drug used to transform either *in vitro* or *in vivo* tumor cells. The tumor cells may be central nervous system cells, lung cells, breast cells, ovary
20 cells, liver cells, squamous cells, prostate cells, pancreas cells, or skin cells. Alternatively, cancer cells from an *in vitro* cultured tumor cell line may be transformed. If the cells are transformed *in vitro*, they may be irradiated so that they are viable but unable to replicate.

For treating cystic fibrosis, the pro-drug is preferably an immuno-
25 modulating drug, such as 15-deoxyspergualin or an antibiotic, such as tobramycin, amiloride or colistine, conjugated with a BLOCK.

For treating chronic nerve pain (neuropathic pain), the pro-drug is preferably an analgesic drug such as an opioid (for example, dihydromorphine, oxycodone, 4'-hydroxyfentanyl or buprenorphine), anticonvulsants (for example,
30 carbamazepine, gabapentin or phenobarbital), tricyclic antidepressant (for example,

desipramine or opipramol), or peptide (for example, Met-enkephalin) conjugated with a BLOCK.

The pro-drug could be used in conjunction with an unconjugated bioactive compound wherein the activation of the pro-drug in the transgenic cells reduces or
5 eliminates the effect of the unconjugated bioactive compound.

The pro-drugs of the invention can be administered using conventional modes of administration including, but not limited to, intravenous, intraperitoneal, oral, intralymphatic, intramuscular, or administration directly into the tumor. Intravenous administration is preferred.

10 The pro-drugs of the invention may be in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic application. For example, oral administration of the β -lactamase conjugates
15 may be disfavored because the conjugates tend to be degraded in the stomach if taken orally, e.g., in tablet form.

The pro-drugs also preferably include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid,
20 potassium sorbate, and salts or electrolytes such as protamine sulfate.

The most effective mode of administration and dosage regimen for the compositions of this invention depends upon the severity and course of the disease, the patient's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the immunoconjugates and pro-drugs should be titrated to the
25 individual patient. Methods of determining dosages are well known in the art.

Nevertheless, an effective dose of the pro-drugs of this invention may be in the range of from about 1.0 to about 1000 mg/Kg/day, the dose of the pro-drug depending upon the particular pro-drug used and the parent bioactive compound from which it is derived. Since the pro-drug is less cytotoxic than the parent bioactive compound, dosages
30 in excess of those recognized in the art for the parent bioactive compound may be used.

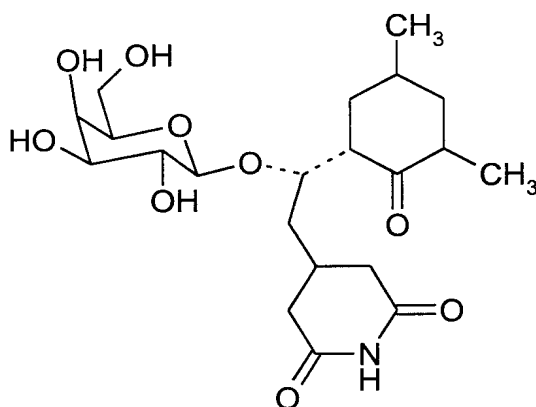
In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting the scope of the invention in any manner.

5 III. Examples.

EXAMPLE 1

PREPARATION OF A CYCLOHEXIMIDE GALACTOSIDE CONJUGATE

The following compound was prepared:



10

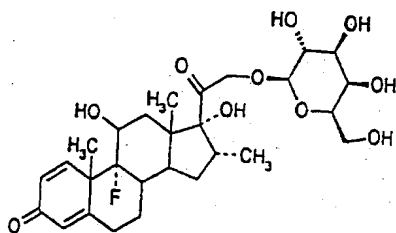
Cycloheximide-galactoside (M0147). An enzymatic method of synthesis was used to produce the β -D-galactoside of cycloheximide (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-O-(β -D-galactopyranosyl)ethyl]glutarimide). Enzymatic galactosylation was carried out using a modification of the procedure of Wong, et al. (1991). Actidione (cycloheximide, 72 mg, 0.256 mmole) and ortho-nitrophenyl β -D-galactopyranoside (132 mg, 0.438 mmole) were dissolved in 0.10 M Na_2HPO_4 /10mM MgCl_2 buffer (pH 7.0, 4 mL) and Tris buffer (pH 7.3, 1 mL) with warming. To this solution was added *E. coli* β -Galactosidase (172 U, 344 μL of a 500 U/mL solution in D.I. water). This mixture was allowed to react at 23°C, for 49 hours until t.l.c. analysis showed production of a lower Rf product (Rf 0.18, 7:3 ethylacetate:methanol irrigant). The reaction mixture was heated to 92°C to destroy the enzyme, lyophilized to a white powder, and chromatographed (silica gel 60A, 25 g) using 0-30% ethylacetate in methanol as eluent. The first major product eluting from the column was combined and

20

evaporated to a white powder (60 mg, 50%). ¹H-n.m.r. (D₂O) was consistent with the proposed structure.

EXAMPLE 2**PREPARATION OF A DEXAMETHASONE GALACTOSIDE CONJUGATE**

The following compound was prepared:



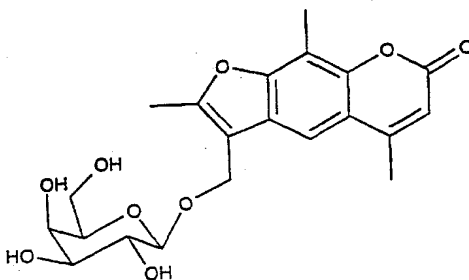
5

Dexamethasone β -D-galactopyranoside (M0149) (21-O- β -D-galactopyranosyl-9-fluoro-11,17-dihydroxy-16-methylpregna-1,4-diene-3,20-dione) was prepared from the parent steroid by treatment with silver triflate/sym-collidine, 3A molecular sieves and acetobromogalactose in dichloromethane:acetonitrile (2:1) under anhydrous conditions in the dark for 20 hours. The fully protected glycoside (M0146) was isolated in 39% yield as the major isomer, which upon deblocking using catalytic sodium methoxide/methanol gave the title compound, in quantitative yield.

10

EXAMPLE 3
PREPARATION OF A HYDROXYMETHYLPSORALEN
GALACTOSIDE CONJUGATE

The following compound was prepared:



5

4'-Hydroxymethyl-trimethylpsoralen was prepared using a modification of the procedure of Isaacs, et al. (Isaacs, S.T., et al., 1977). Under dry conditions, trimethylpsoralen (100 mg, 0.438 mmole) was dissolved in acetic acid (glacial, 10 mL) with heating, cooled to room temperature and chloromethylethylether (0.935 mL, 10 mmole) added with stirring. This mixture was allowed to stir at room-temperature, protected from light, in a sealed flask for 15 hours, after which time additional chloromethylethylether (1.0 mL, 10.8 mmole) was added and the reaction continued until t.l.c. analysis (9:1 chloroform:methanol) showed conversion of the starting material to a lower R_f, blue fluorescent product (R_f 0.92 to 0.68). The reaction mixture was cooled to 0°C (ice-bath), filtered, and the filtrate evaporated to a white powder, which was suspended in distilled water (50 mL) and heated to reflux (3 hours), cooled, and the white precipitate filtered, washed with a minimum ice-cold water, and dried in air. Yield 91 mg (75% of theoretical yield) ¹H-n.m.r. (CDCl₃): δ 2.5-2.6 (9H, m, 3 x -CH₃), 4.8 (2H, s, -CH₂OH), 6.2 (1H, s, H-3), 7.8 (1H, s, H-5).

4'-hydroxymethyl-trimethylpsoralen (44 mg, 0.17 mmole) was dried *in vacuo*, dissolved in anhydrous dichloromethane (20 mL) and placed under a dry nitrogen atmosphere. To this solution was added 3A molecular sieve (0.5 g), acetobromogalactose (140 mg, 0.34 mmole), silver trifluoromethanesulfonate (90 mg, 0.34 mmole)) and sym-collidine (45 μL, 0.34 mmole). This mixture was allowed to stir in the dark at room temperature for 18 hours, filtered through a CeliteTM pad, the precipitate washed with excess chloroform, and the combined filtrates extracted with water, 1N aqueous HCl

25

solution, 2% ammonium hydroxide solution, saturated aqueous sodium bicarbonate solution and water (1x50 mL each). The final organic layer was dried over anhydrous sodium sulfate, evaporated and dried to a white powder, which was subjected to column chromatography (silica gel 60A, 25 grams) using a gradient elution with 0-50% ethylacetate in chloroform as eluent. The first product eluting from the column was combined, evaporated and dried *in vacuo* to give 68 mg (68% of theoretical yield) white powder. ¹H-n.m.r. (CDCl₃) δ: 1.4 (12H, 4s, OCOCH₃x4), 2.4-2.5 (9H, 3s, -CH₃x3), 4.0-4.3 (6H, m, ring protons), 5.0 (2H, m, CH₂O-), 5.2 (1H, d, anomeric H), 6.1 (1H, d, H-3), 7.3 (1H, s, H-5).

10 This dried per-acetate derivative was suspended in anhydrous methanol (30mL) and toluene (2mL) and 25%(w/v) NaOMe/MeOH (200 μL) was added, and this mixture allowed to stir in the dark, under anhydrous conditions for 15 hours. The reaction mixture was neutralized with washed, dry IRC50(H+) resin, filtered, and the resin washed with dry methanol. The combined filtrates were evaporated and dried *in vacuo* to give 41
15 mg of an off white solid (84% of theoretical yield).

EXAMPLE 4

LOSS OF BIOLOGICAL ACTIVITY OF 5-FLUOROURIDINE (5FUR) UPON GALACTOSIDE CONJUGATION. BACTERIAL GROWTH ASSAY

E. coli strains CSH23/S90C and CSH22/S90C were tested with 5-
20 fluorouridine (5FUR) and the 5FUR-galactoside conjugate described in Example 16, 5-fluorouridine-5'-O-β-D-galactopyranoside, (M0174) to confirm loss of biological activity upon conjugation. CSH23 is a strain inducible for *lacZ* expression; CSH22 is a deletion mutant for *lacZ* and is therefore uninducible. Other strains with these properties are commercially available. When grown under non-inducing conditions, neither strain will
25 produce β-galactosidase. Each strain was inoculated into 10 mL M9 minimal media and grown in non-inducing conditions, shaking at 37°C overnight. Overnight cultures (500 μL) were transferred into 10 mL fresh M9 media and grown to logarithmic phase for 4 hours prior to beginning the experiments. 1 mL of this culture was transferred to 10 mL fresh M9 media containing 10 μg/mL 5FUR, 16.2 μg/mL M0174, or no additions, and
30 returned to the incubator. Growth curves were generated by removing aliquots of each culture at 30 minute intervals and reading absorbance at 420 nm. All final data was run in

duplicate trials. As shown in Fig. 1, unmodified 5FUR caused a significant decrease in growth, while the galactoside pro-drug had very little effect, demonstrating loss of biological activity of the compound upon conjugation.

EXAMPLE 5

5 **INDUCTION OF GENE EXPRESSION FOR CONTROL OF PRO-DRUG
RELEASE. RESPONSE OF CSH36 AND W3110 *E. COLI* BACTERIA TO ONPG
APPLICATION WITH OR WITHOUT INDUCTION BY IPTG**

E. coli strains CSH36 and W3110 available from the ATCC, Manassas, VA were tested with orthonitrophenyl β -D-galactopyranoside (ONPG), according to the
10 procedure of Marsh, 1994, to confirm release of compound upon induction of β -galactosidase production. W3110 is a wild-type *E. coli* strain inducible for *lacZ* expression; CSH36 is a constitutive mutant for *lacZ* and therefore produces β -galactosidase regardless of induction conditions. Each strain was inoculated into 10 mL M9 minimal media and grown in non-inducing conditions, shaking at 37°C overnight.
15 W3110 was also inoculated into 10 mL M9 media containing .3 mM iso-propyl thiogalactoside (IPTG) (W3110i) to induce β -galactosidase production. Overnight cultures (500 μ L) were transferred into 10 mL fresh M9 media (+/- IPTG) and grown to logarithmic phase for 4 hours prior to beginning the experiments. 1 mL of this culture was transferred to 10 ml fresh M9 media (+/- IPTG) containing .3 mg/ml ONPG or no
20 additions, and returned to the incubator. Growth curves were generated by removing aliquots of each culture at 30 minute intervals and reading absorbance at 420 nm. All final data was run in duplicate trials (A and B). CSH36 and induced W3110 showed turnover of ONPG to ONP by the development of a yellow color in the media, as ONPG is a colorless compound and ONP is yellow. ONP is also toxic to the bacteria, and
25 therefore growth was retarded in those cultures producing β -galactosidase and turning over ONPG to ONP (see below and Fig. 2).

Strain	IPTG	ONPG	Doubling Time (min)
W3110	no	no	98
W3110	yes	no	88
W3110	no	yes	92
W3110	yes	yes	215
CSH36	no	no	89
CSH36	no	yes	* not measurable

* growth halted approximately 2 hours after start of experiments

EXAMPLE 6

5 **REGULATING SUGAR CONTENT OF MEDIA TO EFFECT DIFFERENTIAL GROWTH OF CREBAG2 CELLS VS. NIH/3T3 CELLS**

Mouse fibroblast cell line NIH/3T3 and its β -galactosidase expressing derivative CREBAG 2 available from ATCC, Manassas, VA, were grown in media containing either only glucose or only lactose to show the ability to select for β -
10 galactosidase expressing cells by providing only lactose as a carbon source, which must be cleaved into glucose and galactose by β -galactosidase before it can be utilized by the cell.

1:1 Dulbecco's Modified Eagles medium (DME): Ham F12 medium was prepared according to standard formula (Freshney) and with the omission of D-
15 glucose, which was replaced by an equimolar quantity of D-lactose. Cell lines were plated in 2, 12 well plates for each cell type, at a density of 5×10^3 cells per well, in DME containing 10% Calf Serum (CS), and incubated for 4 hours at 37°C to allow settling and attachment to the plate. Media was then removed and cells were washed once with
20 phosphate buffered saline solution (PBS). The wells were then filled with the different test medias, including DME:F12 containing glucose and 10% CS (positive control), DME:F12 containing glucose and 10% dialyzed CS (dCS), DME:F12 containing lactose and 10% dCS, and DME:F12 containing no sugar and 10% dCS (negative control).
Dialyzed calf serum was used in place of regular calf serum in the test medias because of the unknown sugar content of the serum . Cells were grown in test medias at 37°C in 5%

CO₂ atmosphere for four days. The MTT assay was performed on one set of plates after two and four days growth to estimate the number of cells and assay growth rates as described in Example 7, below. Doubling times of cells grown in DME:F12 with 10% dCS and either glucose, lactose, or no sugar are listed below. NIH/3T3 cells grown in
 5 lactose showed a doubling time closer to that of cells grown in no sugar than that of cells grown in glucose. CRE BAG 2 cells grown in lactose, however, showed a doubling time very close to cells grown in glucose, showing the ability to select for *lacZ* cells.

Doubling times (days) of cells grown in DME:F12 with 10% dCS

Cell type	no sugar	+ glucose	+ lactose
NIH/3T3	1.09	0.57	0.87
CRE BAG 2	1.35	1.10	1.13

10

EXAMPLE 7

**IN VITRO RESPONSE OF CREBAG2 AND NIH/3T3 CELLS TO 5-FUR-GAL
(M0174) CELL PROLIFERATION ASSAY**

Mouse fibroblast cell line NIH/3T3 and its β -galactosidase expressing derivative CREBAG 2 are plated in 12 well plates at a concentration of 12×10^4 cells per
 15 well and allowed to grow overnight at 37°C, 10% CO₂. Cells are then exposed to various concentrations of M0174 for two hours per day for three days, after which cell proliferation is measured by the MTT assay. Control wells receive only appropriate amounts of solvent used to administer M0174.

For the MTT assay, cells are incubated with the MTT reagent, which is
 20 turned over by live cells to a purple MTT formazan product. Turnover is directly proportional to the number of live cells, enabling a proportional measurement of the number of cells to be achieved by measuring the production of MTT formazan. Media is removed from cells in the twelve well plate and replaced with DME containing 10mM Hepes. .25 mL of a 2 mg/ml solution of MTT is then added to each well, and plates are
 25 covered and wrapped with aluminum foil and incubated at 37°C for three hours. Plates are then emptied of the media MTT solution, and 1 mL of DMSO and 125 μ L of Sorensens glycine buffer are added to each well to solubilize the MTT formazan product. Absorbence of each sample is measured against a DMSO blank at 570 nm.

Growth of cells tested with M0174 are expressed as a percentage of the growth in control wells (absorbance of test well/ absorbance of control well).

EXAMPLE 8

IN VITRO RESPONSE TO 5-FUR-GALACTOSIDE CONJUGATE OF PC5 5 TUMOR CELLS WITH AND WITHOUT RETROVIRAL TRANSFECTION WITH *LACZ*

The PC5 cell line is a model cell line for prostrate tumor cells, and can be transformed with a retroviral vector to produce new cell lines stably expressing β -galactosidase. Viral vector containing the *lacZ* gene and the Neomycin resistance gene
10 (conferring resistance to the selection agent Gentamycin (G418)) is collected in the media from confluent packaging cells producing the virus and filtered through a .45 μ m membrane to remove cellular debris. The viral solution is then added to the media of PC5 cells and incubated overnight. The cells are then split into media containing the selection agent G418 to select for transformants. After 1-2 weeks of G418 selection, the remaining
15 cells are plated in 96 well plates to isolate single cells, and allowed to grow until colonies were several hundred cells. A duplicate plate was created and colonies were tested for β -Gal expression by X-gal staining. Colonies with consistent high levels of expression throughout all cells were chosen for further experiments (PC5 β Gal cells).

PC5 and PC5 β Gal cells are plated in 12 well plates at a concentration of
20 12×10^4 cells per well and allowed to grow overnight at 37°C, 10% CO₂. Cells are then exposed to various concentrations of the 5FUR-galactoside conjugate described in Example 16, 5-fluorouridine-5'-O- β -D-galactopyranoside (M0174) for two hours per day for three days, after which cell proliferation is measured by the MTT assay. Control wells receive only appropriate amounts of solvent used to administer M0174.

25 Growth of cells tested with M0174 are expressed as a percentage of the growth in control wells (absorbance of test well/ absorbance of control well).

EXAMPLE 9

**IN VITRO RESPONSE TO 5-FLUOROURIDINE-5'-O- β -D-
GALACTOPYRANOSIDE OF D5 TUMOR CELLS WITH OR WITHOUT
LIPOFECTION WITH *LACZ* VECTOR**

5 Lipofection of D5 murine melanoma cells with the pCMV β plasmid containing the *lacZ* gene provided a *lacZ* positive set of cells with transient but high levels of β -galactosidase expression. A 60 mm plate of D5 cells, 80% confluent, was lipofected for 5 hours with 3.6 μ g/ml pCMV β (a plasmid containing the *lacZ* gene)(CLONTECH) using 36 μ L lipofectAmine reagent (Life Technologies, Gibco BRL).

10 After lipofection, fresh media was added to the cells to dilute the lipofection reagent, and cells were grown for 2 days. A cover slip of the lipofected D5 cells was stained with X-gal and transfection efficiency was estimated at 70%. D5 and lipofected D5 cells were plated in 12 well plates at a density of 12×10^3 cells per well and incubated 6 hours. The 5FUR-galactoside conjugate described in Example 16, 5-fluorouridine-5'-O- β -D-

15 galactopyranoside (M0174) was added to cells at various concentrations and cells were incubated 6 hours, after which the media was changed. Cells were allowed to grow another 36 hours, then the MTT assay was performed to estimate the number of living cells per well. Cell numbers were expressed as a percent of the number of cells in the control well. All assay points were performed in duplicate and averaged. Administration

20 of M0174 to both lipofected and control D5 cells in culture exhibited excellent differential growth curves with classic dose-response behavior. Lipofected D5 cells, because of *lacZ* expression, activated the M0174 pro-drug and were growth inhibited. Non-lipofected cells were unaffected by M0174 treatment, even at high dosage values.

5FURG (M0174, μ g/ml)	D5 lipofected, % of control growth	D5 % of control growth
0.00000	100.00000	100.00000
0.30000	47.97373	109.89583
5.00000	22.02627	87.94271

EXAMPLE 10**THE *IN VIVO* RESPONSE TO 5-FLUOROURIDINE-5'-O- β -D-GALACTOPYRANOSIDE TREATMENT OF D5 TUMOR CELLS (LIPOFECTED WITH A *LACZ* CONTAINING VECTOR) IN MICE**

5 The 5FUR-galactoside conjugate described in Example 16, 5-fluorouridine-5'-O- β -D-galactopyranoside (M0174) was tested to determine the reduction of tumor growth of subcutaneously implanted D5 mouse melanoma tumors that had been lipofected with a vector containing the *lacZ* marker gene in mice. This data is shown below. C57/J(B6) mice (22-36 g each) were injected in the right flank with 50 μ L HBBS
10 containing 1×10^6 D5 tumor cell that were either untreated or lipofected with a vector containing the *lacZ* gene. M0174 was administered intraperitoneally starting at Day 3, for 4 days, to mice that had received lipofected tumor cells in doses of either 25 or 50 mg/kg/day, and to mice that had received untreated tumor cells in doses of 100 mg/kg/day. Measurement of tumor area in mm^2 began on Day 8 and ended on Day 26.
15 The results clearly show a slight delay in tumor growth at both 25 and 50 mg/Kg/Day groups of mice receiving lipofected tumor cells (5-7 animals/group), indicating drug-targeting *in vivo* by β -galactosidase expressing cells. NO EFFECT (i.e. no significant drug release) for pro-drug on non-lipofected cells was found (even at 100 mg/Kg/Day), indicating once again a lack of biological activity for the pro-drug, M0174. These results
20 show efficacy of pro-drug delivery and efficacy *in vivo*, confirming our *in vitro* results.

EXAMPLE 11**SELECTION OF β -GAL EXPRESSING *E. COLI* CELLS FROM CO-CULTURE BY TREATMENT WITH A GALACTOSIDE PRO-DRUG**

E. coli strains CSH36/S90C and CSH22/S90C were grown in co-culture
25 and tested with the chloramphenicol galactoside pro-drug (M0165) described in Example 15, to show lack of translocation of drug released in β -galactosidase expressing cells to other cells in the culture, and therefore selection for the *lacZ* - cells. CSH36 is a constitutive mutant for *lacZ* and therefore produces β -galactosidase regardless of induction conditions. CSH22 is a deletion mutant for *lacZ* and is therefore uninducible.
30 Each strain individually and both strains together were inoculated into 10 mL aliquots of M9 minimal media and grown shaking at 37°C overnight. Overnight cultures (500 μ L)

were transferred into 10 mL fresh M9 media and grown to logarithmic phase for 4 hours prior to beginning the experiments. 1 mL of this culture was transferred to 10 ml fresh M9 media containing 30 µg/ml chloramphenicol, 42 µg/ml M0165, or no additions, and returned to the incubator. Growth curves were generated by removing aliquots of each culture at 30 minute intervals and reading absorbance at 420 nm. Remaining portions of the cultures were allowed to grow overnight, and final absorbance readings were taken. Serial dilutions of cultures were made in M9 media and plated on M9 plates containing X-gal. Plates were incubated at 37°C for 48 hours, then evaluated for growth of white (indicating *lacZ* -, CSH22 cells) and blue (indicating *lacZ* +, CSH 36 cells) colonies. Co-cultures grown overnight in the presence of either chloramphenicol showed no growth of either cell type, whereas co-cultures grown in the presence of M0165 showed growth of only CSH22 cells, demonstrating selection of the *lacZ*- cell type. This indicates that chloramphenicol was released from M0165 and localized to only the cells expressing β-galactosidase.

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EXAMPLE 12

TRANSLOCATION OF A GALACTOSIDE PRO-DRUG IN *E. COLI*. CREATING A “BYSTANDER” EFFECT BY USING β-GALACTOSIDASE EXPRESSING CELLS

E. coli strains CSH36/S90C and CSH22/S90C were grown in co-culture and tested with the 5FUR-galactoside conjugate described in Example 16, 5-fluorouridine-5'-O-β-D-galactopyranoside, (M0174) to show translocation of drug released in β-Gal expressing cells to other cells in the culture. CSH36 is a constitutive mutant for *lacZ* and therefore produces β-galactosidase regardless of induction conditions. CSH22 is a deletion mutant for *lacZ* and is therefore uninducible. Each strain individually and both strains together were inoculated into 10 mL aliquots of M9 minimal media and grown shaking at 37°C overnight. Overnight cultures (500 µL) were transferred into 10 mL fresh M9 media and grown to logarithmic phase for 4 hours prior to beginning the experiments. 1 mL of this culture was transferred to 10 ml fresh M9 media containing 10 µg/ml 5-fluorouridine (5FUR), 16.2 µg/ml M0174, or no additions, and returned to the incubator. Growth curves were generated by removing aliquots of each culture at 30 minute intervals and reading absorbance at 420 nm. Remaining

portions of these cultures were allowed to grow overnight, and final absorbance readings were taken. Serial dilutions of cultures were made in M9 media and plated on M9 plates containing X-gal. Plates were incubated at 37°C for 48 hours, then evaluated for growth of white (indicating *lacZ* -, CSH22 cells) and blue (indicating *lacZ*+, CSH36 cells)

5 colonies. As indicated in the table below, co-cultures grown overnight in the presence of either 5FUR or M0174 showed no growth of either cell type, indicating translocation of the released drug from the *lacZ*+ CSH36 cells through the media to the *lacZ*- CSH22 cells.

Strain		Drug Added		Colony Growth	
CSH36	CSH22	5FUR	M0174	blue	white
X				X	
X		X			
X			X		
	X				X
	X	X			
	X		X		X
X	X			X	X
X	X	X			
X	X		X		

10

EXAMPLE 13

USE OF PHORBOL OCTANOYL ACETATE GALACTOSIDE OR DI-OCTANOYL RAC-GLYCEROL GALACTOSIDE FOR SELECTIVE GROWTH ENHANCEMENT OF RECOMBINANT MAMMALIAN CELLS

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To test whether the application of growth stimulatory conjugates to recombinant cells can selectively enhance growth with respect to non-transformed cells, Swiss 3T3 cells available from ATCC, Manassas, VA, are transformed by lipofection with the pCMV β plasmid containing the *lacZ* gene provided a *lacZ* positive set of cells with transient but high levels of β -galactosidase expression. A 60 mm plate of Swiss 3T3

20 cells, 80% confluent, is lipofected for 5 hours with 3.6 μ g/ml pCMV β (a plasmid

containing the *lacZ* gene)(CLONTECH) using 36 μ L lipofectAmine reagent (Life Technologies, Gibco BRL). After lipofection, fresh media is added to the cells to dilute the lipofection reagent, and cells are grown in a CO₂-incubator environment (37°C) for 2 days. A cover slip of the lipofected Swiss 3T3 cells is stained with X-gal and transfection efficiency is estimated. Swiss 3T3 and lipofected Swiss 3T3 cells are plated in 12 well plates at a density of 12×10^3 cells per well in DME/Waymouth media containing 2.5% FCS and incubated 6 hours. Phorbol Octanoyl Acetate Galactoside (150 ng/mL) or Di-Octanoyl *rac*-Glycerol Galactoside(40 μ g/mL) are added to one-half of the wells, POA (100 ng/mL) or DOG (30 μ g/mL) to one-eighth of the wells each, and to the remaining wells, no addition (controls). The cells are returned to the incubator and allowed to grow for 6 days as above. Differential growth of the recombinant Swiss 3T3 cells versus non-transformed cells is recorded by measuring the number of cells using the MTT assay (Plumb, et al., 1989).

EXAMPLE 14

15 **USE OF CYCLOHEXIMIDE GALACTOSIDE (M0147) TO SELECTIVELY ABLATE β -GALACTOSIDASE EXPRESSING CREBAG2 CELLS IN CO- CULTURE WITH NIH/3T3 CELLS**

NIH/3T3, CRE BAG 2, or a 50/50 mixture of both cell lines (in co-culture) (Total cell density = 6×10^4 cells each) were plated in 60 mM plates. 12 tissue culture treated glass cover slips were added to each plate, and the cells were allowed to grow in an humidified CO₂-incubator (37°C) for 24 hours. Half the cover slips from each plate were then transferred to separate plates. Cells were treated with 15 μ g/ml cycloheximide β -D-galactopyranoside described in Example 1 (M0147) in media for 14 hours then given fresh media (DME with 10% FCS) for 10 hours. This treatment was repeated twice more, the cover slips were removed from both the M0147 treated and untreated 60 mm plates and placed in 12 well plates. To give an indication of the number of β -galactosidase expressing CREBAG2 cells remaining on the cover slips, the cover slips were incubated with the β -galactosidase substrate ONPG, whose turnover can be measured by the change in absorbance at 420 nm. The cells were rinsed with PBS, then 1 ml of 3.5 mM ONPG in HBSS was added to each well. After 4 hours incubation at 37°C, the solution was removed from each well and the absorbance was measured at 420 nm. Treatment of co-

cultured CREBAG2 and NIH/3T3 cells with M0147 resulted in a decrease of β -galactosidase activity (and therefore the number of CREBAG2 cells) to nearly that of NIH/3T3 cells alone, indicating turnover of the pro-drug in CREBAG2 cells resulting in their selective ablation.

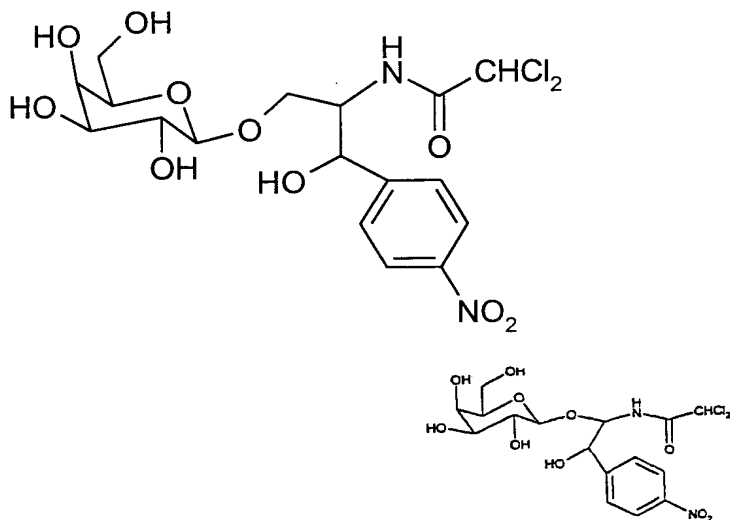
	Growth (abs @ 420 nm)	
	NIH 147 treatment	Control
CRE BAG 2	.450	.561
NIH/3T3	.100	.102
Coculture	.133	.543

5

EXAMPLE 15

PREPARATION OF A CHLORAMPHENICOL GALACTOSIDE CONJUGATE

The following compound was prepared:



10

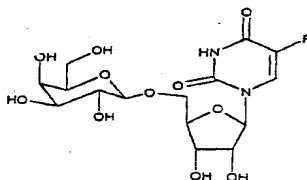
Chloroamphenicol (5.00 g, 15.475 mmole) was dissolved in anhydrous dichloromethane (35 mL) containing 3A molecular sieves, and placed under an atmosphere of dry nitrogen gas. To this solution was added acetobromogalactose (7.00 g, 17.02 mmole), anhydrous silver trifluoromethanesulfonate (4.38 g, 17.02 mmole) and sym-collidine (2.25 mL, 17.02 mmole). This mixture was allowed to stir under anhydrous conditions, in the dark, for 72 hours. Anhydrous acetonitrile (20 mL) was

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added to the flask, and stirring continued as above for 5 hours. The reaction was filtered through a pad of diatomaceous earth (CeliteTM-545) and the precipitate washed with chloroform (5 x 30 mL). The combined filtrates were extracted with water, 1 N hydrochloric acid, saturated aqueous sodium bicarbonate solution, 2% ammonium hydroxide solution, and 5% saturated brine solution (1 x 50 mL each). The resulting organic layer was dried over anhydrous sodium sulfate, filtered, evaporated and applied to a column of silicagel 60A (70-230 mesh, 250g) using chloroform as solvent. The column was eluted by gradient elution from 0% to 4% methanol in chloroform. Fractions of the second major product to elute from the column were combined and evaporated to give the pure protected glycoside (M0160). A 500 mg sample of this material was dissolved in methanol:water (5:1, 60 mL) containing potassium cyanide (88 mg, 1.35 mmole), and this mixture allowed to stir at room temperature for 18 hours at room temperature. The reaction mixture was evaporated to approximately 10 mL, co-evaporated with methanol (4 x 10 mL), never allowing the sample to dry completely. The final sample was dissolved in 10 mL methanol, and dried onto diatomaceous earth (CeliteTM-545) (2 grams). This sample was applied to a column of silicagel 60A (70-230 mesh, 50 g) and eluted with 9:1 chloroform:methanol as eluent. Fractions of the first major product to elute from the column were combined and evaporated to approximately 5 mL, and crystallized by addition of petroleum ether (b.p. = 35-60°C) (100 mL). The white crystalline material obtained was dried *in vacuo* (111 mg, 30%). T.l.c. (SiO₂, irrigant = 8:2 chloroform:methanol) R_f=0.38, ¹H-n.m.r. (D₂O) δ: 8.2 (d, 2H, Ph-H), 7.6 (d, 2H, Ph-H), 6.2 (s, 1H, CHCl₂), 5.2 (d, 1H, Ph-CH(OH)), 4.4 (t, 2H, -CH₂-), 4.2(dd, 1H), 3.9(d, 1H, H-1), 3.8-3.5(m, 6H, ring protons).

EXAMPLE 16
PREPARATION OF A 5-FLUOROURIDINE-5'-O- β -D- GALACTOSIDE
CONJUGATE

The following compound was prepared:



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5-Fluorouridine-5'-O- β -D-Galactopyranoside (M0174) was prepared using a modification of the procedures of Watanabe (Watanabe, K.A., et al., 1981). Under anhydrous conditions, fluorouridine (508 mg, 1.94 mmole) was dissolved in a mixture of anhydrous acetone (15 mL) and 2,2-dimethoxypropane (1.00 mL, 8.13 mmole). To this solution was added para-toluenesulfonic acid, monohydrate (71 mg, 0.37 mmole) and this mixture allowed to stir at room temperature, under an atmosphere of dry nitrogen gas for 18 hours. T.l.c. analysis after this time (SiO₂: irrigant = ethylacetate) showed conversion to a higher R_f product (R_f = 0.69). Solid sodium bicarbonate (114 mg, 1.35 mmole) was added to the solution and stirring continued as above for 2 hours. The reaction mixture was filtered, and the white precipitate washed with acetone (5 x 10 mL). The filtrate was evaporated to a white solid, and dried *in vacuo* (768 mg, crude). This crude product was recrystallized from hot 95% ethanol. After cooling to 4°C overnight, colorless needle crystals of the 2,3-isopropylidene derivative were collected by filtration (366 mg, 63%). A second crop of crystals (113 mg) (total yield 479 mg (82%) were also obtained by concentration of the mother liquors and cooling to 4°C.

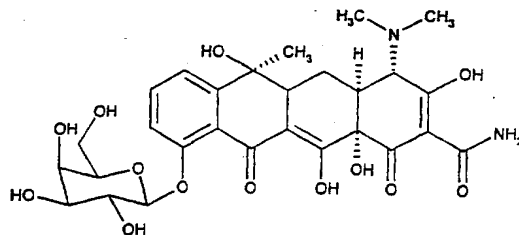
Under anhydrous conditions, the isopropylidene derivative from above (302 mg, 1.0 mmole, 1st crop) was dissolved in anhydrous dichloromethane (30 mL). To this solution was added dry silver oxide (452 mg, 1.95 mmole), resublimed crystalline iodine (30 mg, 0.12 mmole) and acetobromogalactose (452 mg, 1.1 mmole) with stirring. This reaction mixture was heated to reflux, under anhydrous conditions for 18 hours, cooled, and the reaction mixture filtered through a pad of diatomaceous earth (CeliteTM), and the precipitate washed with chloroform (5 x 15 mL). The combined filtrates were

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extracted with water (1 x 50 mL), 0.1 M sodium thiosulfate solution (1 x 50 mL) and water (1 x 50 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness (597 mg, 94%). This fully protected nucleoside derivative (M0172) was deblocked in two steps using, first, the procedure of Lichtenhaler, et al. (Lichtenhaler, et al., 1978). The nucleoside derivative (M0172) was added to a solution of trifluoroacetic acid:water (5:1, 24 mL), and allowed to stir at room temperature for 25 minutes. The solution was then evaporated and co-evaporated with dry toluene (4 X 30 mL) and 1:1 methanol:toluene (1 x 40 mL), and dried *in vacuo* to give the crude peracetate derivative (M0173) (471 mg, grey solid). A sample of this compound (364 mg, 0.54 mmole) was then dissolved in anhydrous methanol (40 mL) under anhydrous conditions (dry nitrogen gas environment) and cooled to 0°C using an ice-bath. Sodium methoxide solution (25%w/v, 500 µL) was added and this mixture allowed to stir at 0°C and at room temperature overnight (18 hours). The reaction mixture was neutralized with washed, dry IRC-50(H+) resin (1 gram). The reaction mixture was filtered, and the resin washed with methanol allowing the washes to equilibrate with the resin during each wash (6 x 30 mL). The combined filtrates were evaporated to a clear oil that was crystallized from methanol:diethylether (1:10, 165 mL) twice. The final crop (201 mg, 87%) was filtered and dried *in vacuo* to an off-white powder. T.l.c (SiO₂, irrigant = 1:1 ethylacetate:methanol, R_f=0.50). ¹H-n.m.r. (D²O) δ: 8.0 (d, 1H, NH), 5.8 (d, 1H, H-1), 4.75 (d, 1H, H-1'), 4.4(d, 1H, H-4'), 4.3-4.1(m, 3H, Gal ring H's), 3.9-3.4 (m, 10H, ring H's).

EXAMPLE 17**PREPARATION OF A TETRACYCLINE GALACTOSIDE CONJUGATE**

The following compound was prepared:



5 Under anhydrous conditions, tetracycline (508 mg, 1.14 mmole) was suspended in anhydrous dichloromethane (25 mL) containing dry 3A molecular sieves (1 gram) with stirring for 1 hour. Dry silver oxide (348 mg, 1.5 mmole), resublimed iodine crystals (96 mg, 0.38 mmole) and acetobromogalactose (630 mg, 1.53 mmole) were added, and the reaction mixture allowed to stir, in the dark, under dry nitrogen gas for 78
10 hours. T.l.c. analysis (SiO_2 : irrigant = 9:1 chloroform:methanol) showed the conversion to three major new products, with R_f values of 0.64, 0.51 and 0.36 respectively, as well as remaining starting tetracycline ($R_f = 0$). The reaction mixture was filtered through a bed of diatomaceous earth (CeliteTM 545), and the precipitate washed with fresh dichloromethane (5 x 20 mL). The combined filtrates were extracted with 50% saturated
15 brine solution (1 x 50 mL), 2% aqueous ammonium hydroxide solution (1 x 50 mL), 1 N aqueous hydrochloric acid solution (1 x 50 mL), and water (1 x 50 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, evaporated, redissolved in chloroform (5 mL) and chromatographed on a column of silicagel 60A (70-230 mesh) (50g), with elution using a gradient from 5% to 20% methanol in chloroform. Fractions
20 from the first major component to elute from the column were combined and evaporated to dryness to give the fully protected glycoside (M0166, 245 mg, 28%).

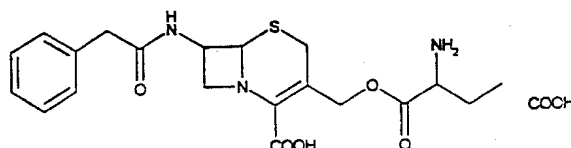
This sample was dissolved in anhydrous methanol (20 mL) containing sodium methoxide (200 μL of a 25% w/v solution in methanol) under anhydrous conditions and allowed to stir at room temperature for 18 hours. The reaction was
25 neutralized with washed, dry IRC50(H^+) resin, filtered and the resin washed with excess methanol (4 x 15 mL) allowing the methanol to equilibrate with the resin for 3 minutes

during each wash step. The combined filtrates were evaporated to dryness to give an off white powder (190 mg, 99% theoretical yield).

EXAMPLE 18

PREPARATION OF A BENZYL CEPHALOTHIN L-GLUTAMATE CONJUGATE

The following compound was prepared:



The title compound was prepared in a four step synthesis using a modification of the methods of Takaya, et al. (Takaya, et al., 1981) and Albrecht, et al. (Albrecht, et al., 1990). 7-Aminocephalosporanic acid (817 mg, 3.0 mmole) was suspended in water (3.3 mL) cooled to 0°C (ice-salt bath), and a solution of 20% (w/v) sodium hydroxide in water (1.4 mL, 7.0 mmole) was added dropwise with stirring over a period of 10 minutes. The starting material was manually stirred until completely dissolved (15 minutes) and the pH adjusted to 8.5 with glacial acetic acid. The reaction mixture was diluted with acetone (2.5 mL) and phenylacetyl chloride (476 µL, 3.6 mmole) added, slowly with stirring. The pH was adjusted to 8.3 with triethylamine (1.5 mL) and this mixture allowed to stir at 0°C for 1 hour. The reaction mixture was evaporated to remove the acetone, the aqueous layer overlayered with ethylacetate (15 mL) and the aqueous layer acidified with concentrated hydrochloric acid solution (pH 3.0), the ethylacetate layer removed, and the aqueous layer extracted with fresh ethylacetate (4 x 20 mL). The combined ethylacetate layers were back-extracted with saturated brine solution (1 x 30 mL) and then dried over anhydrous sodium sulfate. Evaporation gave 870 mg (83% theoretical yield).

Under anhydrous conditions, under dry N_{2(g)}, a sample of this benzyl cephalothin (685 mg, 1.97 mmole) in ethylacetate was treated with freshly prepared diphenyldiazomethane (Org. Synth. Coll. Vol. 3, 351) over a period of 2 hours at ambient temperature until a reddish color persisted in the reaction (total 5.0 mL). After concentrating to 15 mL, and storage at 4°C for 18 hours, the white crystalline precipitate

was collected and dried *in vacuo* (310 mg, 31% theoretical yield). ¹H-n.m.r. (CDCl₃) δ: 7.5-7.2 (m, 15H, Ph-H), 6.9 (s, 1H), 6.1 (m, 1H), 5.9 (m, 1H), 5.0 (dd, 1H), 4.9 (m, 1H), 4.4 (dd, 1H), 3.9 (dd, 1H), 3.7-3.6 (m, 2H), 3.5 (s, 1H), 2.5 (m, 1H).

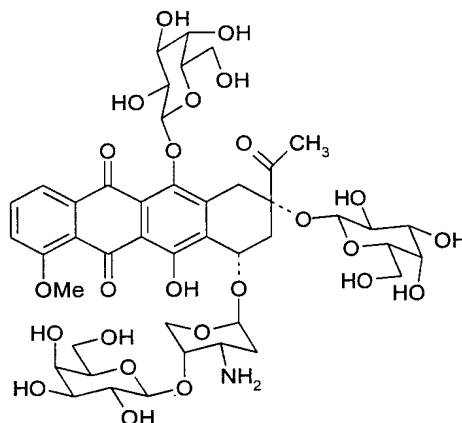
The diphenyl methyl ester, benzyl amide from above (150 mg, 0.29 mmole) was dissolved in anhydrous tetrahydrofuran (10 mL) containing 3A molecular sieves (0.5 g) and cooled to 0°C (ice-bath). N-tert-butyloxycarbonyl-L-glutamic acid g-tert-butyl ester (199 mg, 0.387 mmole), dicyclohexylcarbodiimide (137 mg, 0.664 mmole) and N,N-dimethylaminopyridine (53 mg, 0.44 mmole) were added, and the reaction mixture allowed to stir under anhydrous conditions, under dry nitrogen gas, at ambient temperature for 18 hours after which time t.l.c. analysis showed the appearance of a new product (SiO₂; R_f = 0.40, irrigant = 9:1 chloroform:ethylacetate). The reaction mixture was filtered, and the precipitate washed with chloroform (50 mL); the combined filtrate was extracted with brine solution (50 mL), saturated aqueous sodium bicarbonate solution (50 mL), 1 N aqueous hydrochloric acid solution (50 mL) and brine solution (50 mL). The organic layer was dried over anhydrous sodium sulfate for 18 hours, filtered, evaporated and dried *in vacuo* to a white solid (342 mg) which was applied to a column of silicagel G (70-230 mesh, 25 grams) and eluted with a gradient of chloroform (300 mL), 9:1 chloroform:ethylacetate (500 mL) and 9:1 chloroform:methanol (250 mL). Fractions containing the first major product to elute from the column were combined, evaporated and dried *in vacuo* to a white solid (220 mg, 94% theoretical yield).

Under anhydrous conditions, this sample was partially dissolved in anhydrous anisole (0.66 mL), cooled to 0°C (ice-bath), and treated with anhydrous trifluoroacetic acid (3.3 mL). This homogeneous solution was allowed to stir under these conditions for 4 hours, evaporated and co-evaporated with anhydrous dichloromethane (4 x 20 mL) and anhydrous toluene (20 mL), triturated with ethylacetate (30 mL) for 18 hours at ambient temperature. The white crystalline solid was collected to give 63 mg (50% theoretical yield) of the title compound.

EXAMPLE 19

PREPARATION OF A DOXORUBICIN GALACTOSIDE CONJUGATE

The following compound was prepared:



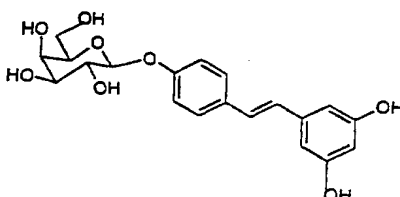
Under anhydrous conditions, doxorubicin (8 mg, 13 μ mole) was dissolved in anhydrous methanol containing triethylamine (2 μ L, 14 μ mole) and trifluoroacetic anhydride (2 μ L, 14 μ mole). This solution was allowed to stir at room temperature, under anhydrous nitrogen gas for 18 hours. The reaction mixture was neutralized with triethylamine (10 μ L) and evaporated and co-evaporated with 1:1 toluene:methanol (3 x 10 mL), and dried *in vacuo*. This solid was suspended in anhydrous dichloromethane (10 mL) containing 3 A molecular sieves (0.5 g), acetobromogalactose (29 mg, 71 μ mole), sym-collidine (9.0 μ L, 67 μ mole) and silver trifluoromethanesulfonate (18 mg, 67 μ mole). This mixture was allowed to stir in the dark, at room temperature under anhydrous nitrogen gas for 120 hours, after which time t.l.c. analysis indicated production of a single product (R_f = 0.53, SiO₂ plate, irrigant = 1:1 acetone:methanol). The reaction mixture was applied directly to a column of silicagel G (70-230 mesh, 10 gram) and eluted with chloroform (50 mL), 9:1 chloroform:methanol (250 mL) and 1:1 acetone:methanol (200 mL). Fractions containing the first major product to elute from the column were combined and evaporated to give the a solid (22 mg, 100% theoretical yield). This sample was dried *in vacuo*, dissolved in anhydrous methanol (10 mL) containing sodium methoxide (71 mg, 1.3 mmole) at 0°C (ice-bath), and allowed to stir at 0°C for 2 hours and at room temperature for 15 hours. The reaction mixture was neutralized with washed, dry IRC-50(H⁺) resin, filtered and the resin washed with methanol (20 mL), water and chloroform to give 21 mg of an orange

solid. T.l.c. analysis (SiO_2 , irrigant = 1:1 acetone:methanol, $R_f = 0.70$), ^1H -n.m.r. analysis conformed to the expected structure for a tri-galactoside conjugate.

EXAMPLE 20

PREPARATION OF A RESVERATROL GALACTOSIDE CONJUGATE

5 The following compound was prepared:



Resveratrol (77 mg, 0.337 mmole) was dissolved in anhydrous dichloromethane (20 mL) and anhydrous acetonitrile (5 mL) containing 3A molecular sieves (1 gram). Sym-collidine (134 μL , 1.01 mmole), dry silver carbonate (140 mg, 0.51 mmole) and acetobromogalactose (416 mg, 1.01 mmole) were added sequentially under anhydrous conditions, and this mixture allowed to stir under anhydrous nitrogen gas, in the dark, for 72 hours at 25°C . T.l.c. analysis (SiO_2 plate; irrigant = 9:1 chloroform:methanol) showed the production of three products ($R_f = 0.53, 0.29, 0.09$ respectively). The reaction mixture was filtered through a CeliteTM or diatomaceous earth pad and the precipitate washed with fresh chloroform (5 x 10 mL). The combined filtrates were extracted with water, 1 N aqueous hydrochloric acid solution, saturated aqueous sodium bicarbonate solution, 2% aqueous ammonium hydroxide solution and water (1 x 50 mL each). The combined organic layers were dried over anhydrous sodium sulfate, filtered, evaporated to dryness and dried *in vacuo*. The crude sample was applied to a column of Silicagel G (70-230 mesh, 20 grams) and eluted with chloroform:methanol (19:1). The second major product to elute from the column was combined, evaporated, and dried *in vacuo* to give a glass (48 mg, 22% theoretical yield). ^1H -n.m.r. analysis (CDCl_3) indicated production of a mono-galactoside product.

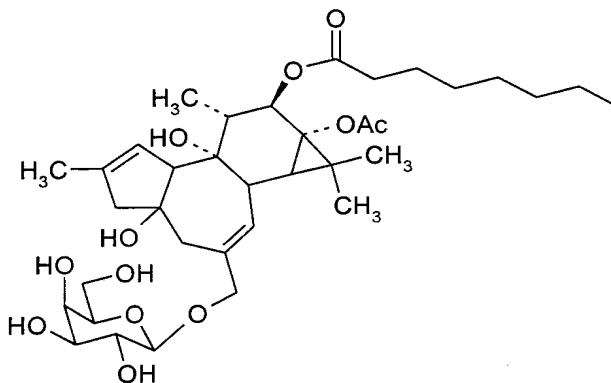
The per-acetate from above (40 mg, 0.07 mmole) is suspended in anhydrous methanol and sodium methoxide/methanol (25% w/v) added (0.5 mL). This mixture is allowed to stir at 0°C for 2 hours and at room temperature for 16 hours. The reaction is neutralized with washed, dry IRC50(H⁺) resin, the resin filtered and washed

with methanol (6 x 10 mL) and the filtrate evaporated to a white solid, homogeneous by t.l.c. (SiO₂: irrigant = 7:3 ethylacetate:methanol).

EXAMPLE 21

PREPARATION OF A PHORBOL OCTANOATE ACETATE GALACTOSIDE CONJUGATE

The following compound was prepared:



Phorbol (4b,9a,12b,13a,20-pentahydroxytigla-1,6-dien-3-one) (40 mg,

0.11 mmole) was suspended in anhydrous acetonitrile (4 mL) containing 3 Å molecular sieves (0.1 g) and acetobromogalactose (55 mg, 0.13 mmole), silver triflate (38 mg, 0.13

10 mmole) and sym-collidine (16 µL, 0.12 mmole) added sequentially, under dry nitrogen gas, slowly with stirring. This mixture was allowed to stir in the dark, under anhydrous conditions for 10 days. After this time, t.l.c. analysis indicated production of two major products (R_f = 0.26 and 0.44; SiO₂ plate: irrigant = 9:1 chloroform:methanol). The reaction mixture was filtered through a pad of diatomaceous earth (Celite 545TM), and the precipitate washed with chloroform (5 x 5 mL) and acetonitrile (5 x 5 mL). To the combined filtrates were added diatomaceous earth (Celite 545TM) (2 grams) and the mixture evaporated to dryness and dried *in vacuo*. This sample was applied to a column of silicagel G (70-230 mesh), slurry packed in chloroform as eluent, and the product eluted by gradient elution using chloroform (200 mL), 9:1 chloroform:methanol (250 mL) and 8:2 chloroform:methanol (250 mL). The first product fractions to elute from the column were combined and evaporated and dried *in vacuo* to give 52 mg (69% theoretical yield) of an off white powder. T.l.c. and ¹H-n.m.r. analysis (d₆-DMSO) indicated that the sample was a mixture of two mono-galactoside isomers.

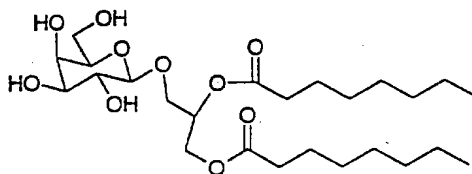
The sample of mixed isomers (52 mg, 75 μ mole) was suspended in anhydrous dichloromethane (3 mL) containing 3 A molecular sieves (0.2 g) and this sample placed under dry nitrogen gas and cooled to 0°C. Dry pyridine (100 μ L, 1.24 mmole) and octanoyl chloride (100 μ L, 586 μ mole, 7.8 equivalents) were added with stirring and the reaction mixture allowed to stir as above at 4°C overnight. The reaction mixture was cooled to 0°C, diluted with dichloromethane (5 mL) and water (10 mL) added. Stirring was continued for 30 minutes, chloroform (50 mL) added and the organic layer separated. This sample was extracted with 1 N aqueous hydrochloric acid solution, saturated aqueous sodium bicarbonate solution, and water (1 x 50 mL each). The final organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness.

One half of this crude sample (37 mg) was dissolved in anhydrous methanol (15 mL), placed under an atmosphere of dry nitrogen gas and cooled to 0°C. Sodium methoxide/methanol solution (25%w/v) (0.25 mL) was added with stirring, and the mixture allowed to stir as above for 2 hours. The reaction mixture was neutralized with washed, dry IRC50 (H+) resin, filtered, the resin washed with methanol (6 x 10 mL) and the combined filtrates evaporated to a off-white waxy solid (40 mg).

EXAMPLE 22

PREPARATION OF A DIOCTANOYL GLYCEROL GALACTOSIDE CONJUGATE

The following compound was prepared:



1,2-isopropylidene *rac*-glycerol was prepared by a modification of the method of Renoll and Newman (Renoll, 1948). Anhydrous glycerol (26.25 g, 0.285 mole) was dissolved in anhydrous acetone (75 mL, 1.02 mole) and petroleum ether (b.p. = 37-58°C) (75 mL) to form two layers. *p*-Toluenesulfonic acid, monohydrate (1.15 g, 6.05 mmole) was added and the reaction mixture heated to reflux using a Dean-Stark trap to collect water produced. After about 7 mL of water was collected in the Dean-Stark trap, the reaction mixture became homogeneous. Refluxing was continued for 13 hours, after

which time approximately 11 mL of water was collected. The reaction was cooled and anhydrous sodium acetate (1.15 grams) was added and the reaction allowed to stir at ambient temperature for 30 minutes, filtered, and the solids washed with petroleum ether (20 mL). The combined filtrates were vacuum distilled (rotary evaporator) to a clear oil (T < 35°C). The final crude product (39.7 grams) was vacuum distilled at 14 mm Hg, and the fraction distilling at 93-95°C collected to give a clear oil (30.98 g, 82% theoretical yield). ¹H-n.m.r. spectral data (d₆-DMSO) was in agreement with the proposed structure.

A solution of the 1,2-isopropylidene glycerol (2.0 g, 15.13 mmole) in anhydrous dimethylformamide (10 mL) was placed under dry nitrogen gas and cooled to 0°C (ice-bath), and benzyl bromide (3.6 mL, 30.26 mmole) added slowly with stirring. Solid dry silver oxide (3.50g, 15.13 mmole) was added slowly with stirring over 30 minutes, and the reaction allowed to stir in the dark, under dry nitrogen gas at ambient temperature for 40 hours. The reaction mixture was then filtered, and the filter cake washed with dimethylformamide (2 x 5 mL) and chloroform (3 x 10 mL). The combined filtrates were extracted with 1% aqueous potassium cyanide solution (100 mL) and the aqueous layer back-extracted with chloroform (3 x 20 mL). The combined organic layers were extracted with water (3 x 20 mL), dried over anhydrous sodium sulfate, filtered, and evaporated. The crude product was adsorbed onto diatomaceous earth (Celite 545TM) (2 g) and applied to a column of Silicagel G (250 grams) slurry-packed using hexanes as solvent, and eluted by gradient elution using 2%, 5%, 10%, 20% and 30% ethylacetate in hexanes (250 mL of each) as eluent. The fractions containing the fourth major product to elute from the column (t.l.c. SiO₂ plate, R_f = 0.20, 5% ethylacetate:hexanes) were combined and evaporated to give 1.69 g (50% theoretical yield) of the title compound. ¹H-n.m.r. (CDCl₃) δ: 7.2 (s, 5H, Ph-H), 4.4 (m, 2H, Ph-CH₂), 4.2 (m, 1H, O-CH), 4.0 (m, 1H, O-CH), 3.6 (m, 1H, O-CH), 3.5-3.3 (m, 2H, 2 x O-CH), 1.3 (s, 3H, CH₃), 1.2 (s, 3H, CH₃).

A sample of the purified benzyl isopropylidene derivative (600 mg, 2.7 mmole) was dissolved in methanol (10 mL) and water (1 mL) with stirring. Solid *para*-toluenesulfonic acid, mono-hydrate (100 mg, 0.53 mmole) was added and the reaction mixture allowed to stir at 25°C for 12 hours and at reflux for 1 hour. After cooling, washed DowexTM 2X4 resin (5 mL, 5 mequiv.) was added and the reaction stirred for 30

minutes. The reaction mixture was filtered and the resin washed with fresh methanol (3 x 10 mL). The combined filtrates were evaporated and dried *in vacuo* to a clear oil (463 mg, 94% theoretical yield), homogeneous by thin layer chromatography (SiO₂ plate, irrigant = 9:1 chloroform:methanol, R_f = 0.33).

5 This sample was converted directly to the di-octanoyl derivative using the procedure of Briggs, et al. (Briggs, et al., 1992). 1-O-Benzyl *rac*-glycerol (463 mg, 2.54 mmole) was dissolved in dry pyridine (10 mL) under anhydrous conditions and dry nitrogen gas, and cooled to 0°C. Redistilled octanoyl chloride (0.96 mL, 5.6 mmole) was added and the reaction mixture allowed to stir as above at 0°C for 2 hours and at room
10 temperature overnight (16 hours). The reaction mixture was poured into saturated aqueous sodium bicarbonate solution (25 mL) and stirred for 10 minutes, and this solution extracted with chloroform (2 x 50 mL). The combined chloroform extracts were back-extracted with water (1 x 50 mL), 1 N aqueous hydrochloric acid solution (2 x 25 mL), saturated aqueous sodium bicarbonate solution (1 x 50 mL) and water (1 x 50 mL). The
15 organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to a clear oil (0.800 g, 72%). T.l.c. (SiO₂ plate, irrigant = chloroform, R_f = 0.19).

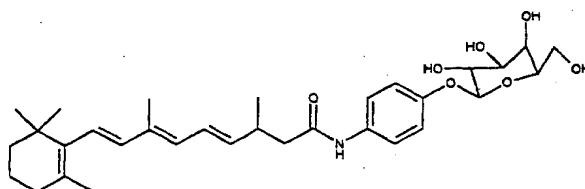
This sample was dissolved in methanol (14 mL) and glacial acetic acid (2 mL), flushed with an atmosphere of hydrogen gas (x3) and 5% palladium on carbon catalyst (0.15 g, 7.5 mg Pd) was added as a slurry in methanol. The reaction mixture was
20 flushed again with hydrogen gas (x3) and left to stir under atmospheric pressure hydrogenation conditions for 7 hours. The reaction was then flushed with nitrogen gas, the catalyst removed by filtering through a pad of diatomaceous earth (CeliteTM 545) and the precipitate washed with methanol (4 x 5 mL). The combined filtrates were evaporated and dried *in vacuo* to yield 620 mg (99% theoretical yield) of a waxy solid. A sample of
25 this material (360 mg) in hexanes (5 mL) was applied to a column of silicagel G (70 -230 mesh, 40 g) slurry packed in 19:1 hexanes:ethylacetate, and the product eluted by gradient elution using 19:1 hexanes:ethylacetate (600 mL), 9:1 hexanes:ethylacetate (200 mL), 17:3 hexanes:ethylacetate (200 mL) and 8:2 hexanes:ethylacetate (200 mL). Fractions containing the second major product to elute from the column were combined and
30 evaporated to give 124 mg of an analytical sample. T.l.c analysis (SiO₂ plate, irrigant = 9:1 chloroform:methanol, R_f = 0.57, irrigant = 4:1 hexanes:ethylacetate, R_f = 0.33).

Enzymatic synthesis of the 3-O- β -D-galactoside was performed using a modification of the procedure of Kren, et al. (Kren, et al., 1992) that employs the β -galactosidase enzyme from *Aspergillus oryzae* reported to provide higher yields of galactosides from lipophilic aglycones.

5 1,2-di-octyl-*rac*-glycerol (DOG) (65 mg, 0.19 mmole) was dissolved in dimethylsulfoxide (100 mL). p-Nitrophenyl β -D-galactopyranoside (ONPG) (150 mg, 0.50 mmole) was dissolved in 0.1 M Na₂HPO₄ / 0.01M MgCl₂ buffer, pH 7.0 (4.0 mL) and Tris buffer (pH 7.3, 1.0 mL) was added. The ONPG solution was added slowly to the DOG solution, with vortex mixing, and the solution warmed to 45°C to dissolve. After
10 cooling, β -galactosidase from *Aspergillus oryzae* (172 U) was added and the reaction mixture slowly rotated at 25°C for 24 hours. The reaction mixture was applied directly to a column of Silicagel G (70-230 mesh, 20g) and eluted by gradient elution using 0-20% methanol in hexanes as eluent. Fractions containing the second major product to elute
15 from the column were combined and evaporated to give a white solid (80 mg, 83% theoretical yield).

EXAMPLE 23
PREPARATION OF A 4-O-GALACTOSYL PHENYL RETINAMIDE
CONJUGATE

The following compound was prepared:



5 p-Nitrophenyl β -D-galactopyranoside (500 mg, 1.659 mmole) was dissolved in dry methanol (30 mL) and a slurry of 5% Palladium on carbon in dry methanol (2 mL, + 2 x 2 mL rinses) added. The reaction flask was evacuated and flushed with hydrogen gas and left to stir under an atmosphere of hydrogen gas for 4 hours. After
10 this time t.l.c. analysis showed the production of a new, ninhydrin positive product ($R_f = 0.62$, SiO_2 ; irrigant = 1:2 chloroform:methanol). ^1H -n.m.r. (D_6 -DMSO) was consistent with the p-aminophenyl β -D-galactoside structure expected.

Under anhydrous nitrogen gas conditions, in the dark, retinoic acid (all-trans) (207 mg, 0.67 mmole) was suspended in dry toluene (5 mL). A solution of
15 phosphorus trichloride (100 μL) in dry toluene (2.5 mL) was prepared in a separate dry flask, under anhydrous conditions, and 2.0 mL of this PCl_3 /toluene solution was added to the reaction (80 mg PCl_3 , 0.88 mmole) in two portions (1.0 mL each) slowly over two hours. The reaction mixture was allowed to react under these conditions for 2.5 hours, after which triethylamine (200 μL , 1.43 mmole) was added to neutralize the excess PCl_3 .

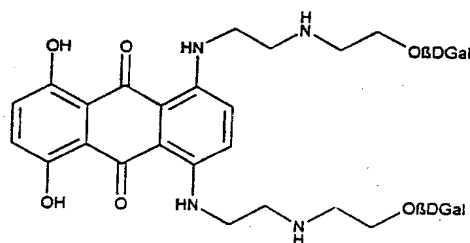
20 A sample of the p-aminophenyl β -D-galactoside was dissolved in N,N-dimethylformamide (5 mL, neutral alumina washed), and the acid chloride solution was added slowly to this by passage through a plug of glass-wool, (+ 2 x 1.0 mL DMF rinses). The reaction was allowed to react at room temperature for 18 hours, and methanol (3.0 mL added to destroy the excess acid chloride. After concentrating to approximately 10
25 mL, the crude product was precipitated by adding diethylether (100 mL), filtered, redissolved in methanol (30 mL) and adsorbed onto diatomaceous earth (CeliteTM 545, 5 grams), and dried *in vacuo*. The sample was purified on a column of silicagel G (70-230 mesh) using a step gradient elution method (9:1; 8:2; 7:3; 6:4; and 1:1

chloroform:methanol; 200 mL each) as eluent. The second major product fractions to elute from the column ($R_f = 0.64$; SiO_2 : irrigant = 7:3 chloroform:methanol) were combined, evaporated and dried *in vacuo* to give a yellow solid (53 mg, 14% theoretical yield). ^1H -n.m.r. (d_6 -DMSO) δ : 9.9 (s, 1H, N-H), 7.5 (d, 2H, Ph-H), 6.9 (d, 2H, Ph-H), 6.4-5.9 (m, 4H, C=C-H), 5.1 (d, 1H, -OH), 4.8 (d, 1H, -OH), 4.7 (d, 1H, H-1), 4.6 (m, 1H, -OH), 4.5 (d, 1H, -OH), 3.7 (d, 1H, H-4), 3.5-3.1 (m, 5H, ring protons), 2.3 (s, 3H, -CH₃), 2.0 (m, 2H, CH₂), 2.0 (s, 3H, -CH₃), 1.7 (s, 3H, -CH₃), 1.5 (m, 2H, CH₂), 1.4 (m, 2H, CH₂) and 1.0 (s, 6H, 2 x -CH₃).

EXAMPLE 24

PREPARATION OF A MITOXANTRONE DI-GALACTOSIDE CONJUGATE

The following compound was prepared:



Method A: Under anhydrous conditions and a dry nitrogen gas atmosphere, mitoxantrone, di-hydrochloride salt (40 mg, 77 μmole) was suspended in dry dichloromethane (20 mL) containing sym-collidine (40 mL) and 3 Å molecular sieves (0.3 g). Acetobromogalactose (196 mg, 0.48 μmole), silver triflate (123 mg, 0.48 μmole) and additional sym-collidine (120 mL, 0.91 μmole) were added, and the reaction flask stirred in the dark for 4 days. The reaction mixture was applied directly to a column of silicagel G (70-230 mesh, 25g) slurry-packed in chloroform, and eluted with 9:1 chloroform:methanol (250 mL) and 1:1:0.1:0.1 ethylacetate:methanol:triethylamine:water (300 mL). Fractions containing the first bluish product to elute from the column were combined and evaporated to give the crude peracetylated galactoside (142 mg).

This sample was dried *in vacuo* overnight, over P_2O_5 . It was dissolved in anhydrous methanol (20 mL) cooled to 0°C , and 25% (w/v) sodium methoxide/methanol (500 mL, 2.19 μmole) added and allowed to stir at 0°C for 2 hours and at 25°C for 14 hours. The reaction mixture was neutralized with washed, dry IRC50 (H⁺) resin (1 gram) and the resin filtered and washed with fresh methanol (20 mL) chloroform (20 mL) and

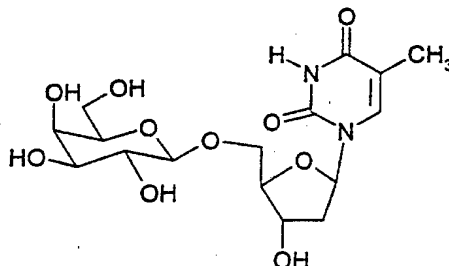
water (20 mL). The combined filtrates were evaporated and dried *in vacuo* to a blue glass (75 mg) that was to a preparative t.l.c. plate (Silicagel GF: 20x20 cm, 1 mm thickness) and eluted with 1:1 acetone:methanol. The product band ($R_f=0.75-0.85$) was removed and the product eluted from the silica using 1:1 acetone:methanol. The eluted product
5 fraction was filtered, evaporated and recrystallized from methanol:diethylether (1:10) to give a blue-white powder (9 mg, 20%). ^1H -n.m.r. analysis was consistent with the structure as shown.

Method B: p-Nitrophenyl β -D-galactopyranoside (150 mg, 0.50 mmole) was dissolved in 0.1 M Na_2HPO_4 / 0.01M MgCl_2 buffer, pH 7.0 (4.0 mL) and Tris buffer
10 (pH 7.3, 1.0 mL) was added. Mitoxantrone, dihydrochloride salt (50 mg, 0.11 mmole) was added, and the mixture vortex mixed for 3 min. *E. coli* β -Galactosidase (172 μL of a 500 U/mL solution in water, 86 U total) was added and the solution rotated slowly at 25°C for 24 hours. T.l.c. analysis (SiO_2 plate, irrigant = 8:2:1:0.1 isopropanol:water:triethylamine:acetic acid) indicated product formation. Purification is
15 carried out by preparative t.l.c using the similar systems to those employed in Method A above, to give the title compound.

EXAMPLE 25

PREPARATION OF A THYMIDINE 5-O- β -D-GALACTOSIDE CONJUGATE

The following compound was prepared:



5 Under anhydrous conditions, thymidine (200 mg, .826 mmoles) was dissolved in anhydrous dichloromethane (10 mL). To this solution was added acetobromogalactose (845 mg, 2.05 mmoles), silver carbonate (170 mg, .620 mmoles), and sym-collidine (220 μ L, 1.65 mmoles) with stirring. This reaction mixture was stirred in the dark under anhydrous conditions for 24 hours. The reaction mixture was then
10 filtered through a pad of diatomaceous earth (CeliteTM), and the precipitate washed with chloroform (5 x 15 mL). The combined filtrates were extracted with water (1 x 100 mL), 2% ammonium hydroxide solution (1 x 100 mL), 1.0 N Hydrochloric acid solution (1 x 100 mL), saturated sodium bicarbonate solution (1 x 100 mL), and water again (1 x 100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated
15 to dryness. The sample was then subjected to column chromatography (silica gel 60A, 35 grams) using a gradient elution with 0-40% ethyl acetate in chloroform as eluent. The second major product eluting from the column was combined, evaporated and dried *in vacuo* to give 42 mg (9% theoretical yield). T.l.c. (SiO₂, R_f=0.53, irrigant = 1:1 chloroform:ethylacetate). ¹H-n.m.r. (CDCl₃) δ : 7.8(s, 1H), 5.6-5.3 (m, 4H), 5.2 (dd, 1H, H-3'), 5.1(m, H-4'), 4.5-4.0 (m, 5H), 2.9 (m, 1H, H-5), 2.2 (m, 2H, CH₂), 2.2-2.0 (4 x s, 3H each, -Oac). 1.6 (s, 3H, -CH₃).

This dried per-acetate derivative was dissolved in anhydrous methanol (10 mL) and cooled to 0°C. 25% (w/v) NaOMe/MeOH (100 μ L) was added, and this mixture allowed to stir under anhydrous conditions for 15 hours. The reaction mixture was
25 neutralized with washed, dry IRC50(H⁺) resin, filtered, and the resin washed with dry methanol. The combined filtrates were evaporated to a small volume (5 mL), to which

was added diethyl ether (30 mL). The crystallized product was filtered, redissolved in MeOH, then evaporated and dried *in vacuo* to give a white crystalline solid (18 mg, 60% theoretical yield), homogeneous by t.l.c. (SiO₂, R_f = 0.68, irrigant = 9:1 ethylacetate:methanol).

5

EXAMPLE 26

PHASE I CLINICAL STUDY INVOLVING ADMINISTRATION OF MARKER GENE MODIFIED OVARIAN TUMOR CELLS WITH SUBSEQUENT PRO-DRUG THERAPY

The maximum tolerated dose (up to 10¹⁰ cells per treatment) of genetically modified tumor cells (see Example 10) will be determined. Tumor cells from patients will be removed by biopsy, and grown *in vitro* using standard clinical tissue culture techniques. Once stable growth in culture is confirmed, transformation of these cells with a vector containing the *lacZ* gene will be effected using either a lipofection technique (see Example 9) or a retroviral vector technique. Four patients will be injected intra-tumorally, with 3 x 10⁷ irradiated tumor cells on day 0. Approximately 24 hours after cell infusion, the patients will be started on a 14 day course of I.V. pro-drug administration. Two weeks after the termination of pro-drug therapy, each patient will be evaluated in terms of side-effects from treatment, and progression of the disease state. If no adverse toxicity occurred from the previous tumor cell treatment and pro-drug therapy, and there appears to be no evidence of progressing tumor growth that would require alternate therapy, then the patient will be eligible for the next dose (regimen) of cellular/pro-drug therapy. This schedule will continue for doses of 1 x 10⁸ and 3 x 10⁸ cells for Group 1. If no adverse side effects warranting the stoppage of therapy are reported in Group 1, then Group 2 patients will be treated at one dose level higher, and this pattern will continue through four groups. If a patient has to drop out of the study for reasons other than toxicity, then another patient may be added to the group and will begin therapy at the lowest dose level for that group.

The patients will be admitted to the hospital for the first fifteen days of each cycle. The transformed cells will be given on day 1 and pro-drug treatment on days 2 through 15. The treatment is repeated at three week intervals for a total of three cycles

30

of treatment. At the completion of treatment, patients will be followed regularly until, and if there is a progression of disease.

Cellular and pro-drug administration. Patients will be assigned in order of entrance in the study to one of the four treatment schedules below. The dosages escalate
 5 with each treatment unless toxicity of Grade II or higher has occurred. For Grade II toxicity, except for neutropenia or thrombocytopenia, the dose is repeated but physician discretion could lower the dose if overall toxicity is of concern. For Grade III or Grade IV toxicity, the dosage is lowered one level.

Patients	First Dose	Second Dose	Third Dose
1-4	3×10^7	1×10^8	3×10^8
5-8	1×10^8	3×10^8	1×10^9
9-12	3×10^8	1×10^9	3×10^9
13-16	1×10^9	3×10^9	1×10^{10}

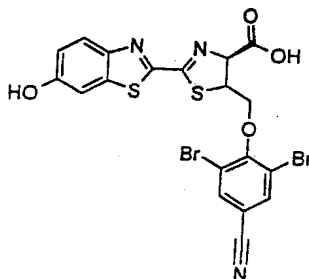
10 The cell suspension is prepared in 1.5 cc of normal saline and administered through a small intratumoral catheter which is inserted on the day of treatment and removed after the 1 hour infusion. Catheter placement and diffusion of fluid will be checked by a technician flow study prior to cell infusion.

The drug conjugates are all prepared from FDA-approved drugs used for
 15 treatment of human cancers. The bioavailability and clearance levels of pro-drugs are required to be measured prior to treatment. The pro-drugs will be provided as a sterile powder and they are reconstituted with sterile water. For I.V. administration, these will be prepared in 100 cc of normal saline, or 5% dextrose and water, and infused over a period of one hour.

20 The standard dose of these drugs will be from 0.5 to 5 mg/kg b.i.d. when patients have a creatine clearance > 80. If the creatine clearance level is 50-79, the dose is reduced to by one-half to 0.25-2.5 mg/kg b.i.d. Daily CBC and platelet counts will be obtained during treatment. The pro-drug administrations should be stopped if the absolute granulocyte count falls below 750 or the platelets are less than 50,000 count.

EXAMPLE 27**PREPARATION OF A D-LUCIFERIN-BROMOXYNIL CONJUGATE.**

The following compound is prepared:



5 **Method A: Preparation of a 5-Oxymethyl-D-Luciferin Cycloheximide Conjugate**

5-Hydroxymethyl-D-Luciferin. A modification of the procedure of Seto, S., et al., 1963 is used. A mixture of 2-Cyano-6-hydroxybenzthiazole (200 mg), 5-hydroxymethyl D-Cysteine (200 mg), potassium carbonate (160 mg) and 50% aqueous methanol (10 mL) is stirred under a stream of nitrogen gas, at room temperature for 2.5
10 hours. After neutralization with 1 N aqueous HCl, the precipitate is collected, washed with water and recrystallized from 95% ethanol to give the title compound.

6'-O-t-butyldimethylsilyl-5-hydroxymethyl-D-Luciferin, t-butyldimethylsilyl ester. Under anhydrous conditions. 5-hydroxymethyl-D-Luciferin (45mg, 0.145 mmole) is dissolved in anhydrous dimethylformamide (7 mL), cooled to
15 0°C (ice-bath) and imidazole (300 µL) and t-butyldimethylsilyl chloride solution (50% solution in tetrahydrofuran, 52 µL, 2.1 equiv.) is added. The reaction mixture is allowed to stir at 0°C for 2 hours and at room temperature overnight. The major product is purified by silicagel column chromatography using dichloromethane:ethylacetate gradient elution. Fractions containing the first major product to elute from the column are
20 combined and evaporated to give the title compound as a white solid.

5-O-(cycloheximidyl)oxymethyl-D-Luciferin. Under anhydrous conditions, 6'-O-t-butyldimethylsilyl-5-hydroxymethyl-D-Luciferin, t-butyldimethylsilyl ester (54 mg, 0.10 mmole) is dissolved in dichloromethane (150 mL). A solution of carbonyldiimidazole (17 mg, 0.105 mmole) in dichloromethane (10 mL) is added slowly
25 over 25 minutes. The reaction mixture is allowed to stir at room temperature for 1 hour, and the volume reduced to approximately 20 mL (rotary evaporator, and a solution

containing cycloheximide (28 mg, 0.10 mmole) in dichloromethane (10mL) is added. This final solution is allowed to stir at room temperature for an additional 6 hours. The reaction mixture is poured directly onto a column of 50 grams (100 mL) silicagel and eluted using a dichloromethane:ethylacetate gradient elution. Fractions containing the conjugate product from the column are combined and evaporated to give a protected compound as a white solid. The final product is deprotected using a dilute solution of tetrabutylammonium fluoride in methanol to give the title compound.

Method B: Preparation of a D-Luciferin-Bromoxynil Conjugate.

Potassium-L-threonate. A modification of the procedure of Humphlett (Humphlett, W. J., 1967) is employed. Oxygen is bubbled through a stirred solution of potassium hydroxide (112 g) in water (240 mL) and methanol (1 L) employing a gas dispersion tube. A solution of L(+)-arabinose (100 g, 666 mmol) in water (240 mL) is added to the stirred, oxygenated solution at such a rate that a temperature of 30-35°C is maintained. Oxygen was continuously bubbled during the addition and for an additional hour after the addition is complete. Air was then bubbled through the solution for an additional 48 hours. The solution is diluted with methanol (1.6 L) and stored in a freezer for several days. The resulting solid is collected, washed with methanol and dried to yield the title compound as a white solid (75 g, 65% theoretical yield).

Methyl 2,4-dibromo-2,4-dideoxy-L-threonate. A modification of the procedure of Bock, et. al. (Bock, et al., 1983) is employed. A solution of potassium-L-threonate (20.0 g, 115 mmol) in 32% hydrogen bromide in acetic acid is stirred at room temperature for 24 hours. Methanol (500 mL) is stirred in and the solution allowed to stand overnight to give a suspension. The solid (potassium bromide) is removed by filtration and the solvent removed from the filtrate. Methanol is added to the residue (200 mL) and the mixture heated at reflux for 2 hours. The methanol is removed and the residue dissolved in ethyl acetate (100 mL). The solution is washed with water (100 mL) and dried over sodium sulfate. The solvent is removed and the residue crystallized from ether/pentane to yield the title compound (24.0 g, 76%).

Methyl (2*S*,3*S*)-4-acetoxy-2,3-epoxybutanoate. The general procedure of Manchand, et. al. (Manchand, et al., 1988) is employed. Methyl 2,4-dibromo-2,4-dideoxy-L-threonate (131 g, 474 mmol) is added to a stirred solution of potassium acetate

(102 g, 1.04 mol) and potassium iodide (3.94 g, 23.8 mmol) in dimethyl formamide (500 mL). This mixture is stirred under nitrogen gas at 50-55°C for 4 hours and overnight at room temperature. The reaction mixture is diluted with ethyl acetate (1 L) and filtered. The filter cake is washed with ethyl acetate (250 mL). The combined ethyl acetate
5 portion is washed with saturated sodium bicarbonate solution (500 mL) and saturated brine (2 x 500 mL) and dried over magnesium sulfate. The solvent is removed to yield the title compound as a dark oil (82.0 g, 99% theoretical yield).

(2*S*,3*S*)-2,3-Epoxy-4-hydroxybutanoic acid sodium salt. A modification of the general procedure of Manchand, et. al. (Manchand, et al., 1988) is employed. A
10 solution of methyl (2*S*,3*S*)-4-acetoxy-2,3-epoxybutanoate (81.8 g, 465 mmol) in methanol (450 mL) is cooled in an ice bath to 5°C. The solution is treated with solid sodium hydroxide at such a rate that the temperature remained less than 15°C. This mixture is stirred at 5°C for 3 hours and diluted with dichloromethane (200 mL). The mixture is stirred at room temperature for 30 minutes. The solid is collected by filtration and
15 washed with dichloromethane (200 mL). The resulting filter cake is dried *in vacuo* at room temperature to yield the title compound as a solid (58.8 g, 90% theoretical yield).

Methyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3,4-dihydroxybutanoate. The general procedure of Manchand, et. al. (Manchand, et al., 1988) is employed. A solution of (2*S*,3*S*)-2,3-epoxy-4-hydroxybutanoic acid sodium salt (58 g, 414 mmol) in ammonium
20 hydroxide (600 mL) is stirred at 50-55°C for 45 hours. The solvent is removed and the residual water is removed as an azeotrope with toluene (400 mL) to give a dark oil. The oil is dissolved in methanol (400 mL) and the solution is cooled to 5°C. A solution of anhydrous HCl (50 g) in methanol (400 mL) is added to the stirred solution over 20 minutes. The solution is stirred overnight at room temperature and the solvent is removed
25 to give a brown oil. The oil is dissolved in methanol (500 mL) and the solution is cooled to 5°C. The cold solution is treated with 1 N NaOH until the pH is approximately 8 as determined universal pH paper. The solution is diluted to a total volume of 1 L with water and sodium bicarbonate (53.4 g, 636 mmol) is added. Neat di-*t*-butyl dicarbonate (126 g, 576 mmol) is added so that the reaction temperature remains below 30°C. After
30 addition, the reaction is stirred overnight at room temperature. A portion of the methanol (ca. 450 mL) is removed by evaporation and the concentrate is cooled to 5°C. The cold

concentrate is diluted with ether and the mixture is stirred at 5°C for 30 minutes. The resulting solid is collected by filtration and washed with water (200 mL) and cold (5°C) ether. The filter cake is dried to yield the title compound as a solid.

(2*R*,3*R*)-2-(*t*-BOC-amino)-3,4-dihydroxybutanoic acid. A solution of methyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3,4-dihydroxybutanoate is dissolved in 95% ethanol and an excess of 2 N potassium hydroxide is added. The solution is stirred overnight at room temperature and the ethanol is removed by evaporation. The pH of the resulting solution is carefully raised to 4 by the addition of 10% hydrochloric acid. The mixture is extracted with ethyl acetate. The extract is washed with water and brine and dried over sodium sulfate. The solvent is removed to yield the title compound.

2,4,6-Trimethylbenzyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3,4-dihydroxybutanoate. A mixture of (2*R*,3*R*)-2-(*t*-BOC-amino)-3,4-dihydroxybutanoic acid, 2,4,6-trimethylbenzyl bromide (1.1 eq.) and triethyl amine (1.1 eq.) in dimethylformamide is stirred overnight at 50°C. The reaction is allowed to cool to room temperature and added to water. The mixture is extracted with ethyl acetate. The extract is washed with water and brine and dried over sodium sulfate. The product is purified by chromatography on silica gel with methanol/dichloromethane elution. The solvent is removed from the pure product fractions to yield the title compound.

2,4,6-Trimethylbenzyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3-hydroxy-4-tetrahydropyranyloxybutanoate. A solution of 2,4,6-trimethylbenzyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3,4-dihydroxybutanoate (1 eq.) in dry dichloromethane is prepared at room temperature. The solution is cooled to -20°C and pyridinium-*p*-toluenesulfonate (0.1 eq.) is added followed by dihydropyran (1.1 eq.). After addition, the reaction is stirred overnight at -20°C. The solution is washed with water and brine and dried over magnesium sulfate. The solvent is removed to yield the title compound.

2,4,6-Trimethylbenzyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3-mesyloxy-4-tetrahydropyranyloxybutanoate. The general procedure of Manchand, et. al. (Manchand, et al., 1988) is employed. A solution of 2,4,6-trimethylbenzyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3-hydroxy-4-tetrahydropyranyloxybutanoate (1 eq.) is prepared in 1,2-dimethoxyethane. The solution is cooled to -20°C and triethyl amine (3 eq.) is added. Methanesulfonyl chloride (2.4 eq.) is added drop wise to the stirred solution. The reaction mixture is

stirred for an additional hour at -20°C and saturated brine is slowly added. The mixture is extracted with ethyl acetate. The extract is washed with saturated brine and dried over magnesium sulfate. The solvent is removed and the residue is crystallized to yield the title compound.

5 2,4,6-Trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-tetrahydropyranyloxy-3-(triphenylmethylmercapto)butanoate. A solution of 2,4,6-trimethylbenzyl (2*R*,3*R*)-2-(*t*-BOC-amino)-3-mesyloxy-4-(tetrahydropyranyloxy)butanoate (1.0 eq.), triphenylmethylmercaptan (2.0 eq) and triethyl amine (2.0 eq) in DMF is stirred at 80 °C for 24 hours. The reaction is allowed to cool to room temperature and added to water.
10 The mixture is extracted with ethyl acetate. The ethyl acetate portion is washed with water and brine and dried over sodium sulfate. The solvent is removed. The residue is purified on by chromatography on silica gel with ethyl acetate hexanes elution. Solvent removal from the pure fractions yields the title compound.

 2,4,6-Trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-hydroxy-3-
15 (triphenylmethylmercapto)butanoate. A solution of 2,4,6-trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-tetrahydropyranyloxy-3-(triphenylmethylmercapto)butanoate in acetic acid: tetrahydrofuran: water (4:2:1) is stirred at 45°C for 3.5 hours. The solvent is removed to yield the title compound.

 2,4,6-Trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-mesyloxy-3-
20 (triphenylmethylmercapto)butanoate. A solution of 2,4,6-trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-hydroxy-3-(triphenylmethylmercapto)butanoate (1.0 eq.) is prepared in 1,2-dimethoxyethane. The solution is cooled to -20°C and triethyl amine (3.0 eq.) is added. Methanesulfonyl chloride (2.4 eq.) is added drop wise to the stirred solution. The reaction mixture is stirred for an additional hour at -20°C and saturated brine is slowly
25 added. The mixture is extracted with ethyl acetate. The extract is washed with saturated brine and dried over magnesium sulfate. The solvent is removed and the residue is purified by chromatography on silica gel with ethyl acetate hexanes elution. The solvent is removed from the pure product fractions to yield the title compound.

 2,4,6-Trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-(2,6-dibromo-4-
30 cyanophenyl)oxy-3-(triphenylmethylmercapto)butanoate. A solution of 2,4,6-trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-mesyloxy-3-

(triphenylmethylmercapto)butanoate (1.0 eq), 2,6-dibromo-4cyanophenol (2.0 eq) and potassium carbonate (2.0 eq) in DMF is stirred at 80°C for 24 hours. The reaction is allowed to cool to room temperature and added to water. The mixture is extracted with ethyl acetate. The extract is washed with water and brine and is dried over sodium sulfate. The solvent is removed and the residue is purified by chromatography on silica gel with ethyl acetate in hexanes elution. The solvent is removed from the pure product fractions to yield the title compound.

(2*R*,3*S*)-2-Amino-4-(2,4-dibromo-4-cyanophenyl)oxy-3-mercaptobutanoic acid trifluoroacetate. A solution of 2,4,6-trimethylbenzyl (2*R*,3*S*)-2-(*t*-BOC-amino)-4-(2,6-dibromo-4-cyanophenyl)oxy-3-(triphenylmethylmercapto)butanoate (1 eq.) and phenol (4 eq.) in trifluoroacetic acid is heated at reflux for 30 minutes. The reaction is allowed to cool to room temperature and is diluted with water. The solution is extracted with ether and the solvent is removed from the aqueous phase. The residue is crystallized from ethanol to yield the title compound.

D-2-(6'-Hydroxybenzothiazol-2'-yl)-D2-5-(2,6-dibromo-4-cyanophenoxy)methylthiazoline-4-carboxylic acid. A solution of 2-cyano-6-hydroxybenzthiazole (2.0 eq.) in MeOH is purged with nitrogen for 5 minutes. A solution of (2*R*,3*S*)-2-amino-4-(2,4-dibromo-4-cyanophenyl)oxy-3-mercaptobutanoic acid trifluoroacetate (1.0 eq.) and potassium carbonate (1.0 eq.) in deoxygenated water is added to the methanol solution. The reaction is stirred under nitrogen while being protected from light for 2 hours. The volume of the reaction is doubled with water and the resulting solution is made acidic with dilute hydrochloric acid. The mixture is extracted with ethyl acetate. The extract is washed with water and dried over sodium sulfate. The solvent is removed and the residue is triturated with ether. The residue is dried *in vacuo* to yield the title compound.

EXAMPLE 28

TISSUE SELECTIVE ABLATION OF MERISTEM FLORAL INITIATOR CELLS IN *LUC* TRANSFORMED *ARABIDOPSIS THALIANA*

To test for induction of sterility and prevention of cross-pollination of recombinant plants, *Arabiposis thaliana* ecotype Columbia are transformed with a plasmid construct containing an *LEAFY* promoter (*LFY*) driven firefly luciferase (*luc*)

gene by standard leaf disk techniques (Horsch, et al., 1988). The 2.3 kb BamHI fragment spanning the *LFY* promoter (Blazquez, et al., 1997) is inserted in front of the *luc* gene in plasmid pGL3 (available from Promega Corp., Madison, WI) using the BglII site.

Transgenic plants are generated using the vacuum-filtration method of Berthold, et al.

- 5 (Berthold, et al., 1993). Regenerated kanamycin-resistant T1 plants carrying *LFY::luc* are grown up in a greenhouse to allow seed to set. T2 seed of strongly expressing T1 lines are grown in sterile culture on MS medium containing kanamycin at 20°C for 6 to 10 days in 16 hours light: 8 hours dark cycles and afterward in 12 hour light: 12 hour dark cycles. Plants are treated by spray application to the upper leaves and apical shoot sections, with
- 10 5 mM D-luciferin-bromoxynil in 0.01% aqueous triton X-100, 2 times per day, beginning on day 4, for a period of 7-20 days. Analysis of the apical shoot meristem of these treated recombinant plants indicate no transition to floral initiation during this time period. Control untransformed plants grown under identical conditions exhibit induction of floral initiation tissues in the apical meristem by microscopic and visual examination.

EXAMPLE 29:**POSITIVE SELECTION OF *LACZ* TRANSFORMED MAMMALIAN CELLS
USING GROWTH-REGULATORY GALACTOSIDE CONJUGATE ADDITION.**

Murine fibroblast cell lines NIH/3T3 and leukemia virus transfected
 5 CREBAG2 (*lacZ*+) were obtained from the ATCC cell repository (Rockville, MD) as
 frozen stocks, and were grown in culture (37°C, 10% CO₂, humidified incubator) using
 1:1 DME:F12 media to establish stable growth. These cells were trypsinized, diluted to 5
 x 10³ cells/mL in DME:F12 media and plated into twelve-well culture plates (Falcon
 #3043). After overnight incubation the media was changed to a minimal F12:DME media
 10 with four components (myo-inositol, pantothenic acid, pyridoxine, and thymidine)
 omitted. The table below indicates the component mixture for this minimal media used.

Components of 1:1 DME:F12 Minimal Media

(Components to weigh directly to 1 L Dist. D.I. water)

Name	Weight
Calcium Chloride -2H ₂ O	154.5 mg
Magnesium Chloride- 6H ₂ O	61.2 mg
Magnesium Sulfate (anhy.)	48.84 mg
Potassium Chloride	311.8 mg
Sodium Chloride	6.996 g
Sodium phosphate Dibasic	71.02 mg
Sodium Phosphate Monobasic	54.3 mg
L-Alanine	4.45 mg
Arginine HCl	147.5 mg
Asparagine	7.5 mg
Aspartic Acid	6.65 mg
Cystine-HCl-H ₂ O	17.56 mg
Cystine · 2HCl	31.29 mg
Glutamic Acid	7.35 mg
Glutamine	365 mg

Glycine	18.75 mg
Histidine-HCl-H ₂ O	31.48 mg
Isoleucine	54.47 mg
Leucine	59.05 mg
Lysine-HCl	91.25 mg
Methionine	17.24 mg
Phenylalanine	35.48 mg
Proline	17.25 mg
Serine	26.25 mg
Threonine	53.45 mg
L-Tryptophan	9.02 mg
Tyrosine	38.7 mg
Valine	52.85 mg
Choline Chloride	8.98 mg
Folic Acid	2.66 mg
HEPES	3.57 mg
Phenol Red	8.1 mg
Pyruvic Acid-Na salt	55 mg
insulin	10 mg
transferrin	25 mg

(Components to Add from Solution)

Name		[Initial]	Volume Added/Liter
Cupric Sulfate-5H ₂ O	1.3 µg/L	13 mg/L	100 µL
Ferric Nitrate-9H ₂ O	50 µg/L	50 mg/100 mL	100 µL
Ferrous Nitrate-7H ₂ O	417 µg/L	41.7 mg/100 mL	1 mL
Zinc Sulfate-7H ₂ O	432 µg/L	43.2 mg/100 mL	1 mL
D-Biotin	3.5 µg/L	35 mg/L	100 µL
Riboflavin	219 µg/L	21.9 mg/100 mL	1 mL
Vitamin B-12	680 µg/L	68 mg/100 mL	1 mL

Linoleic Acid	42 µg/L	100 mg/23.8 mL	100 µL
Putrescine- HCl	81 µg/L	81 mg/100 mL	100 µL
Thioctic Acid	105 µg/L	105 mg/100 mL	100 µL
Niocrinimide	2.02 mg/L	101 mg/50 mL	1 mL
Pyridoxal	2 mg/L	101 mg/50 mL	1 mL
Thiamine-HCl	2.17 mg/L	108.5 mg/50 mL	1 mL
Hypoxanthine	2.1 mg/L	105 mg/50 mL	1 mL

Note: All components added prior to dilution to 1.0 L.

Cell media were prepared starting from the base minimal F12:DME and adding (1) glucose (0.63 g/L), (2) lactose(1.26 g/L), (3) glucose (0.63 g/L) and myo-
 5 inositol β -D-galactoside (0.239 g/L), pantothenic acid di- β -D-galactoside (3.76×10^{-3} g/L), thymidine β -D-galactoside (6.09×10^{-4} g/L) and pyridoxine β -D-galactoside (6.07×10^{-5} g/L), (4) lactose (1.26 g/L) and myo-inositol β -D-galactoside (0.239 g/L),
 pantothenic acid di- β -D-galactoside (3.76×10^{-3} g/L), thymidine β -D-galactoside (6.09×10^{-4} g/L) and pyridoxine β -D-galactoside (6.07×10^{-5} g/L), or nothing (blank). Cells were
 10 allowed to grow in these media for 1-4 days, and the number of viable cells counted using the MTT assay (Plumb, et al., 1989). After 3 - 4 days in culture, the differential growth of the CREBAG2 (*lacZ*⁺) cells versus NIH/3T3 cells (*lacZ*⁻) was obtained. These observations indicate that other combinations of growth-factor conjugates, application dynamics, or cell lines may also be used for positive selection of *lacZ*⁺ cells in culture.

	NIH3T3 Relative Cell Growth	CREBAG2 Relative Cell Growth
Glc + 4V	0.0925	0.1560
Lac + 4VGal	-0.0124	0.0269
Glc + 4VGal	0.0078	0.0300
Glc only	0.00	0.00

EXAMPLE 30**DIFFERENTIAL GROWTH OF AMP-CONTAINING RECOMBINANT BACTERIA BY APPLICATION OF AN AMPICILLIN CONJUGATE.**

Xanthomonas strains were kindly provided by Don Crawford, Culture
5 Curator for the Nutrasweet Kelco Company (San Diego, CA). Appropriate permits for
transport of these strains were obtained from the USDA. *X. Campestris* XN1 is a
nalidixic acid-resistant derivative of *X. campestris* NRRL B-1459S-4L. *X. campestris*
CL355 is an XN1 derivative containing plasmid pGC9114, a plasmid containing the *lac*
transposon Tn951, and conferring resistance to ampicillin, kanamycin, and tetracycline
10 (Walsh, *et. al*, 1984).

Strains were reconstituted from freeze dried cultures by addition of 1 ml of
Luria-Bertani glucose (LBG) broth. Suspended cultures were then added to nutrient agar
slants and incubated at 30°C until growth was observed. Individual colonies of these
strains were then grown and maintained in LBG broth.

15 *X. campestris* strains XN1 and CL355 were grown in M9 minimal media
plus 0.2% glucose. The glutamate-ammonia ligase inhibitor phosphinothricin (PPT) was
added to three XN1 and CL355 cultures at a concentration of 5 µM. L-glutamate (20
µg/ml) was added back to one XN1 and CL355 culture containing 5 µM PPT.
Phenylacetyl-cephalosporanic acid-glutamate (M0182)(63 µg/ml, equimolar to 20 µg/ml
20 L-glutamate) was added to XN1 and CL355 cultures containing 5 µM PPT. Control
cultures containing no additions were also included. Growth of cultures was monitored
by recording change in absorbance at 600 nm over time. Differential growth of strains
containing the ampicillin-glutamate derivative is noted for the recombinant bacteria (*X.*
campestris CL355) versus non-transformed strain XN1. The final growth results in media
25 of M9+0.2%glucose+ PPT (20 µg/mL)+M0182(63 µg/mL) were 0.471 at Abs.(600 nm)
for CL355 and 0.101 at Abs. (600 nm) for XN1.

EXAMPLE 31**THE *IN VIVO* RESPONSE TO DOXORUBICIN-GALACTOSIDE TREATMENT OF TUMOR CELLS LIPOFECTED WITH A *LACZ* CONTAINING VECTOR IN MICE**

5 To test the efficacy of pro-drug delivery *in vivo* for initiation and mediation of a host cellular immune response to genetically modified tumor, C57/J(B6) mice (22-36g each) are injected injected intravenous with 50 μ L HBBS containing 1×10^6 D5 tumor cells that are either untreated, lipofected with a vector containing the *lacZ* gene, lipofected with a vector containing both the *lacZ* and the H-2K allogeneic major
10 histocompatibility complex (MHC) class I gene (Wahl, et al, 1995), or lipofected with a vector containing the H-2K gene alone. After 2 days, accumulation of the tumor cells in the lung tissue is expected. Two animals are retained as controls, and necropsy examination and tumor staining of the lipofected animals is performed at the end of the trial, to confirm pulmonary tumor growth.

15 The doxorubicin-galactoside pro-drug of Example 19 is administered intraperitoneally starting at Day 3, for 20 days, to mice that received lipofected tumor cells in doses of either 5, 10 or 25 mg/kg/day, and to mice that received untreated tumor cells in doses of 50 mg/kg/day. Measurement of tumor growth is determined by necropsy examination of lung tissue and staining at day 25. Tumor regression is noted in lipofected
20 animals versus tumor growth in those animals receiving the non-transformed tumor cells. In addition, those animals receiving both *lacZ* and H-2K gene exhibit lower tumor burden, presumably due to a host cellular immune response, initiated by the selective drug delivery.

EXAMPLE 32

25 **PHASE I CLINICAL STUDY INVOLVING ADMINISTRATION OF THE *LACZ* MARKER GENE TO DELTAF508 CFTR DEFECTIVE LUNG EPITHELIAL CELLS WITH SUBSEQUENT PRO-DRUG THERAPY.**

In vivo transformation of deltaF508 CFTR defective lung epithelial cells with a vector containing the *lacZ* gene will be effected using either a lipofection technique
30 (see Canonico, A.E., Plitman, J.D., Conary, J.T., Meyrick, B.O., Brigham, K.L., J. Appl. Physiol. 1994 77(1): 415-419) or a retroviral vector (see Yoshimura, K., Rosenfeld, M.A.,

Seth, P., Crystal, R.G., J. Biol. Chem. 1993 268(4): 2300-2303) technique. Four patients will be subjected to *lacZ* transformation on day 0. Approximately 24 hours after cell transformation, the patients will be started on a 10 day course of I.V. pro-drug administration. Two weeks after the termination of pro-drug therapy, each patient will be evaluated in terms of side-effects from treatment, and progression of the disease state. If no adverse toxicity occurred from the previous cell treatment and pro-drug therapy, and there appears to be no evidence of increased immune response that would require alternate therapy, then the patient will be eligible for the next regimen of cellular/pro-drug therapy. This schedule will continue for regimens of 1, 3 and 5 repetitions for Group 1. If no adverse side effects warranting the stoppage of therapy are reported in Group 1, then Group 2 patients will be treated at one dose level higher, and this pattern will continue through four groups. If a patient has to drop out of the study for reasons other than toxicity, then another patient may be added to the group and will begin therapy at the lowest dose level for that group. The patients will be admitted to the hospital for the first two days of each cycle. The transformation of cells will be performed on day 1 and pro-drug treatment on days 2 through 15, with day 3-15 pro-drug treatment on an out-patient basis. The treatment is repeated at three week intervals for a total of one to five cycles of treatment. At the completion of treatment, patients will be followed regularly until, and if there is a progression of disease for bacterial infection, CFTR function, and pulmonary function.

Cellular transformation and pro-drug administration. Patients will be assigned in order of entrance in the study to one of the four treatment schedules below. The dosages escalate with each treatment unless toxicity of Grade II or higher has occurred. For Grade II toxicity, except for neutropenia or thrombocytopenia, the dose is repeated but physician discretion could lower the dose if overall toxicity is of concern. For Grade III or Grade IV toxicity, the dosage is lowered one level. The drug conjugates are all prepared from FDA-approved drugs used for treatment of human cancers. The bioavailability and clearance levels of pro-drugs are required to be measured prior to treatment. The pro-drugs will be provided as a sterile powder and they are reconstituted with sterile water.

For I.V. administration, these will be prepared in 100 cc of normal saline, or 5% dextrose and water, and infused over a period of one hour. The drugs to be administered are 15-Deoxyspergualin β -D-galactoside and Genistein- β -D-galactoside for CFTR membrane expression regulation, tobramycin β -D-galactoside for reduction of pulmonary lung bacterial infection, and the bronchodilator albuterol β -D-galactopyranoside to improve pulmonary function.

The standard dose of these drugs will be from 0.5 to 10 mg/kg b.i.d. when patients have a creatine clearance > 80. If the creatine clearance level is 50-79, the dose is reduced to by one-half to 0.25-2.5 mg/kg b.i.d. Exact drug levels and combination drug therapy will be determined at the time of treatment. Daily CBC and platelet counts will be obtained during treatment. The pro-drug administrations should be stopped if the absolute granulocyte count falls below 750 or the platelets are less than 50,000 count.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

EXAMPLE 33

SELECTION OF *lacZ* TRANSFORMED MAMMALIAN CELLS USING A CYCLOHEXIMIDE-GALACTOSIDE CONJUGATE TO INHIBIT CELL ADHESION

Murine fibroblast cell lines NIH/3T3 and leukemia virus transfected CREBAG2 (*lacZ*+) were obtained from the ATCC cell repository (Rockville, MD) as frozen stocks, and were grown in culture (37°C, 10% CO₂, humidified incubator) using Dulbecco's Modified Eagles (DME) media containing 10% calf serum (CS) to establish stable growth. NIH/3T3, CREBAG2 or a 50:50 mixture of both cell lines (co-culture) were grown to approximately 70% confluence as above and treated with either cycloheximide (80 μ L of a 5 mg/mL solution per 10 mL of media), cycloheximide-b-D-galactopyranoside (M0147, 600 μ L of a 5 mg/mL solution per 10 mL of media) or no-drug (controls) and incubated for 20 min. prior to trypsinizing and counting.

These cells were plated out into 6-well tissue culture plates (CoStar #3516) at a density of 2×10^5 cells/mL (2.0 mL/well) and treated with either cycloheximide (15

uL of a 5 mg/mL solution per well, [37.5 μ g/mL]), cycloheximide-b-D-galactopyranoside (M0147, 125 μ L of a 5 mg/mL solution per well, [312.5 μ g/mL]) or no-drug (controls) and incubated in DME media as above for 2 hours. The plates were then slowly rocked (American Rotator V Model R4140, Speed = 56 rpm) for 30 min. in the incubator as
5 above, and the supernatant media and cells were removed by suction into sterile 2.5 mL eppendorf tubes, spun at 2000 rpm for 3 min. (Eppendorf centrifuge model 5417C), the top layer of media removed, and the cell pellet resuspended in fresh media (2.0 mL). Fresh media (2.0 mL) was also added to each well of the remaining (adherent) cells in the plates, and the plates returned to the incubator for 3 hours prior to assay. The supernatant
10 (selected) cells were plated into new 6-well plates and also incubated for 3 hours. After this time, standard X-Gal staining (according to the procedures of Lojda, 1970 and Sanes, et al, 1986) shows approximately 50% enrichment of lacZ+ cells in the selected cell plates obtained from co-cultured cells and M0147 (cycloheximide-b-D-galactopyranoside) treatment versus controls.

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EXAMPLE 34

CONTROL OF GENE EXPRESSION BY RELEASE OF DEXAMETHASONE FROM *lacZ* TRANSFORMED MAMMALIAN CELLS BY TREATMENT WITH A DEXAMETHASONE-GALACTOSIDE CONJUGATE

A mammalian cloning vector containing Green Fluorescent Protein
20 sequence under control of a dexamethasone inducible promoter was constructed as follows. The pMSG vector (Amersham-Pharmacia, 10 μ L of a 0.5 μ g/mL solution) was linearized by incubation with the endonuclease SmaI (Roche, Indianapolis, IN, 1 μ L of a 10U/ μ L solution) in 0.1 M Tris-acetate buffer containing 10 mM MgOAc and 0.5 mM DTT (1.5 μ L) and DI H₂O (2.5 μ L) to a total volume of 15 μ L, for 3 hours at 25°C. The
25 reaction products were run on a 0.8% agarose gel, and the major product (4.95 kb) was excised from the gel and purified using a silicagel membrane like the QIAquick membrane (Qiagen, Valencia, CA) and eluted with 10 mM Tris-Cl buffer, pH 8.5 (50 μ L).

The pEGFP-1 vector (Clontech, Palo Alto, CA; 10 μ L of a 0.5 μ g/mL
30 solution) was linearized by incubation with the endonuclease SmaI (Roche, Indianapolis, IN; 1 μ L of a 10U/ μ L solution) in 0.1 M Tris-acetate buffer containing 10 mM MgOAc

and 0.5 mM DTT (1.5 μ L) and DI H₂O (2.5 μ L) to a total volume of 15 μ L, for 3 hours at 25°C. The reaction products were run on a 0.8% agarose gel, and the major product (4.2 kb) was excised from the gel and purified using a silicagel membrane like the QIAquick membrane (Qiagen, Valencia, CA) and eluted with 10 mM Tris-Cl buffer, pH 8.5 (50 μ L).

The gel purified linearized vectors (50 μ L each) were further incubated with the endonuclease HindIII (1 uL of a 10 U/ μ L solution), in 10 mM NaCl, 5 mM MgCl₂, 1 mM 2-mercaptoethanol, pH 8 and DI H₂O to a reaction volume of 60 μ L for 18 hours at 37°C. The reaction products were run on a 0.8% agarose gel, and the major products (4.2 kb for pEGFP-1 and 1.46 kb for pMSG) were excised from the gel and purified using a silicagel membrane like the QIAquick membrane (Qiagen, Valencia, CA) and eluted with 10 mM Tris-Cl buffer, pH 8.5 (50 uL for the pEGFP-1 fragment and 30 uL for the pMSG fragment). The concentration of each vector fragment was estimated as 5 ng/uL for the promoter and 20 ng/uL for the pEGFP-1 vector using a 1/HindIII -1kb ladder on a 0.8% agarose gel.

Ligation of 2.5 μ L of cut pEGFP-1 vector and 3.5 μ L of promoter fragment from pMSG were incubated with 1 Unit T4 DNA ligase (Promega, Madison, WI) at 23°C for 24 hours, in a total volume of 10 μ L. Competent JM109 *E. Coli* cells were transformed with 2.5 μ L of ligations by heat shock method of Hanahan (Hanahan, 1983), and 100 μ L of transformations were plated on LB – ampicillin plates and incubated overnight at 37°C. Colonies from plates were inoculated into 1.4 ml terrific broth and incubated shaking at 37°C overnight. Plasmid DNA was purified from cultures using a spin miniprep and cut with BglII and SmaI enzymes to confirm presence of promoter insert. (1 μ L miniprep DNA was incubated with 1 unit SmaI endonuclease enzyme (Roche, Indianapolis, IN) in 0.1 M Tris-acetate buffer containing 10 mM MgCl and 0.5 mM DTT for 24 hours at 25°C. Reactions were heated to 65°C for 20 min. to inactivate SmaI enzyme. 1 unit BglII enzyme (Roche) was added to all reactions and incubated 24 hours at 37°C). The new plasmid was named pDex-GFP.

3T6 cells (obtained from the ATCC cell repository, Rockville, MD) were grown to approximately 50 % confluence and transformed with pDex-GFP as follows. 1 ug of DNA was diluted with 50 uL of DME media (without CS). 6 μ L Lipofectamine

solution (Gibco-BRL) was combined with 50 μ L DME w/o serum. DNA and lipid solutions were mixed and incubated 15 min at room temperature. Cells were rinsed with DME w/o serum and 400 μ L DME w/o serum was added to each well of cells. The DNA/lipid solutions were added to the cell media, mixed, and incubated 5 hrs at 37°C.

- 5 Transfection media was then removed and replaced with RPMI + 10% calf serum. The pDex-GFP cells were grown to 70% confluency, trypsinized and replated at 4×10^5 cells/mL concentration in 60 mm plates containing 10 mm sterile cover slips.

- Dexamethasone (15 μ L of a 40 μ g/mL solution in 1% ethanol/RPMI) was added and the cells incubated (37°C, 10% CO₂, humidified incubator) overnight. Cells
10 were fixed (3% paraformaldehyde in PBS) on coverslips and examined using a Nikon fluorescence microscope (Nikon Labophot-2, Nikon, Inc., Melville, NY). pDex-GFP/3T6 cells exhibited a green fluorescence when excited at 488 nm. pEGFP-1 transfected 3T6 cells exhibited no fluorescence under identical conditions.

Dexamethasone induction between CREBAG2 and pDex-GFP/3T6 cell lines.

- 15 CREBAG2, pDex-GFP/3T6 or a 50:50 mixture of both cell lines (co-culture) are grown to approximately 70% confluence in 60 mm culture plates (37°C, 10% CO₂, humidified incubator) and treated with either dexamethasone (15 μ L of a 40 μ g/mL solution in 1% ethanol/RPMI), dexamethasone b-D-galactopyranoside (M0149, 20 μ L of a 50 μ g/mL solution in PBS), or no drug (control). After incubation overnight (37°C, 10%
20 CO₂, humidified incubator) and fixation (using 3% paraformaldehyde in PBS) the cells are examined using a Nikon fluorescence microscope (Nikon Labophot-2). The pDex-GFP/3T6 –CREBAG2 (co-culture) plates treated with dexamethasone b-D-galactopyranoside (M0149) exhibit a green fluorescence when excited at 488 nm.

EXAMPLE 35
CELL SURFACE EXPRESSION OF E. coli *lacZ* IN MAMMALIAN CELLS FOR
EXTRACELLULAR RELEASE OF HIGH-MOLECULAR WEIGHT
CONJUGATES

5 A cloning vector containing a signal sequence like the Murine Ig kappa-chain V-J2-C at the amino terminus to target proteins to the plasma membrane, and a transmembrane domain than can be fused to the carboxy terminus to anchor proteins to the membrane, was used to construct *lacZ* fusions. The *lacZ* gene was subcloned in frame with this transmembrane domain. The pDisplay vector (Invitrogen, Calsbad, CA)
10 represents such a vector. The pDisplay vector also contains an HA epitope tag that can be used to detect protein expression using fluorescent antibody staining and the gene for neomycin resistance useful in selection with geneticin, disulfate salt (antibiotic G418). These sequences are not necessary for practice of the invention.

Subcloning of *lacZ* into a pDisplay vector. Method A:

15 Primers designed to create appropriate restriction sites were generated to amplify *lacZ* from the pcDNA3.1/His/*lacZ* vector (Invitrogen, Calsbad, CA). Forward primer for *lacZ* (gaagatctcgggatctgtacgac), and reverse primer for *lacZ* (tcccccgaggacaccagaccaact) were purchased from SIGMA-Genosys (St. Louis, MO). Amplification of *lacZ* was performed in three, 50 μ L reactions, with 200 ng plasmid
20 DNA, 0.2 mM dNTPs, 10 mM Tris pH 9, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 2 U Taq polymerase, and 1 μ M each of forward and reverse primers. A thermocycler (like the Techne Genius Thermal Cycler, Princeton, NJ) was programmed for the following cycles: 1.) 94°C, 4 min. 2.) 42°C, 2 min. 3.) 72°C, 2 min. 4.) 74°C, 45 sec. 5.) 69°C, 45 sec. 6.) 72°C, 2 min. 7.) 34X steps 4 – 6. 8.) 72°C, 5 min. Reactions
25 were run on a 0.8% agarose gel, and the major amplification product (3 kb) was excised from the gel and purified using a silicagel membrane like the QIAquick membrane (Qiagen, Valencia, CA) and eluted with 10 mM Tris-Cl buffer, pH 8.5.

 10 μ g pDisplay vector and the purified *lacZ* product was cut with 10 Units SmaI endonuclease enzyme (Roche, Indianapolis, IN) in 0.1 M Tris-acetate buffer
30 containing 10 mM $MgCl$ and 0.5 mM DTT for 24 hours at 25°C. Reactions were heated to 65°C for 20 min. to inactivate SmaI enzyme. 10 Units BglII enzyme (Roche) was

added to all reactions and incubated 24 hours at 37°C. Reactions products were run on a 0.8% agarose gel and bands were excised and gel purified as above.

50 ng cut pDisplay vector and 57 ng of *lacZ* insert were incubated with 1 Unit T4 DNA ligase (Promega, Madison, WI) at 23°C for 24 hours, in a total volume of 10 µL. Competent *E. Coli* cells were transformed with 5 µL of ligations by heat shock method of Hanahan (Hanahan, 1983), and 100 µL of transformations were plated on LB – ampicillin plates and incubated overnight at 37°C. Colonies from plates were inoculated into 1.4 mL terrific broth (TB) and incubated shaking at 37°C overnight. Plasmid DNA was purified from cultures using a spin miniprep and cut with BglII and SmaI enzymes as above. Plasmids that showed proper sized fragments after digestion were sequenced to confirm proper reading frame of the insert. The new plasmid was named pZDisplay.

Subcloning of *lacZ* into a pDisplay vector. Method B:

5 µg pcDNAHis *lacZ* (Invitrogen) was cut with 5 Units Kpn I and 5 units Bpu 1102I (MBI Fermentas) in a total reaction volume of 20 µL using 33 mM Tris-acetate buffer (pH 7.9) containing 10 mM magnesium acetate, 66 mM potassium acetate and 0.1 mg/ml BSA, incubating overnight at 37°C.

6 µg pDisplay (Invitrogen) was cut with 5 units SmaI (Roche Biochemicals) in a total reaction volume of 10 µL with 33 mM Tris-acetate buffer (pH 7.9) containing 10 mM magnesium acetate, 66 mM potassium acetate and 0.5 mM dithiothreitol, incubating overnight at 25°C. Reactions were ethanol precipitated and resuspended in 5 µL TE (10 mM Tris-Cl, 1 mM EDTA)

The cut pDisplay vector was treated with alkaline phosphatase to prevent religation of vector. 2.5 µg of cut pDisplay was incubated with 2 units alkaline phosphatase (Roche Biochemicals) in a total reaction volume of 27 µL with 50 mM Tris-HCl, 0.1 mM EDTA (pH 8.5) for 90 minutes at 37°C. 2.5 µL 200 mM EGTA was added to stop the reaction. Reaction was heated 10 min at 65°C to inactivate phosphatase.

pcDNAHis*lacZ* reaction was blunted by a fill in reaction with T4 DNA polymerase (2 units) in a total reaction volume of 20 µL with 0.1 mM dNTPs, 50 mM Tris-HCl, 15 mM (NH₄)₂SO₄, 7 mM MgCl₂, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.02 mg/ml BSA (pH 8.8) incubating at 37°C for 15 minutes, then stopping the reaction by incubating at 75°C for 10 min.

The above reactions were run on a 0.75% agarose gel and the cut, phosphorylated pDisplay vector, and the blunted 3 kb lacZ insert were excised and gel purified.

Ligations were performed using T4 DNA ligase (1 unit) in reaction
5 volumes of 10 μ L using 27 ng cut, phosphorylated pDisplay vector and 42 ng lacZ insert with 66 mM Tris-HCl, 5 mM MgCl₂, 1 mM dithioerythritol, and 50 μ g/mL BSA, incubating overnight at room temperature.

JM105 competent cells were transformed with the ligations by the heat shock method of Hanahan, and 100 μ L aliquots of transformations were plated on LB
10 ampicillin plates. Colonies were inoculated into 1.5 ml TB and grown overnight shaking at 37°C. Plasmids were purified from cultures using method of Sambrook, et al. (Sambrook, 1989).

Plasmids were digested with the endonuclease Bgl II (Roche) and run on a
0.8% agarose gel. Plasmids of appropriate size were sequenced to confirm orientation
15 and frame of insert.

Expression of pZDisplay in D5 murine tumor cells.

D5 cells were plated in 12 well plates and allowed to grow to 50% confluence. 1 μ g of DNA (pZDisplay, or pcDNA/his/lacZ) was combined with 50 μ L DME w/o serum. 8 μ L Lipofectamine solution (Gibco-BRL) was combined with 50 μ L
20 DME w/o serum. DNA and lipid solutions were mixed and incubated 15 min. at room temperature. Cells were rinsed with DME w/o serum and 400 μ L DME w/o serum was added to each well of cells. The DNA/lipid solutions were added to the cell media, mixed, and incubated 5 hrs (37°C, 10% CO₂, humidified incubator). Transfection media was then removed and replaced with RPMI + 10% calf serum. After two days growth,
25 G418 was added to the media at 0.7 mg/ml. Cells were maintained in selection media for two weeks and then plated in 96 well plates at a dilution of 1 cell/well. Cells were allowed to grow until colonies could be identified, then colonies were transferred to 12 well plates containing sterile 10mm cover slips.

Clones were assayed for expression of *lacZ* by immunohistochemical
30 staining. Cells were fixed in 2.5% paraformaldehyde and permeabilized in microtubule stabilizing buffer with 0.5% Triton X-100. Cells were blocked in PBS with 0.3% BSA

and incubated with mouse anti-HA or anti- β -galactosidase antibody (Sigma, St. Louis, MO) diluted 1:100 in PBS with 0.3% BSA for 1 hr at room temperature, then washed in PBS and incubated with FITC conjugated anti-mouse antibody (Sigma) for 2 hrs at room temperature. Two clones showed good staining: D5 + pZDisplay B7 and D5 + pZDisplay B12.

pZDisplay clones were also assayed for β -galactosidase activity by incubation with ONPG. pZDisplay clones and control D5 and D5A2 (stable *lacZ* expressing) cells were plated in 12 well plates at approximately 2×10^5 cells/well. Cells were allowed to attach to plates, then media was removed and replaced with 0.5 mg/ mL ONPG in DME without phenol red or calf serum. Cells were incubated overnight and then ONPG media was removed from each well and the absorbance was read at 420 nm. (see Table below.) Cells remaining in the plate were quantified by the MTT assay to estimate relative β -galactosidase activity/cell.

15

20

	ONPG	MTT	TURNOVER/CELL
E1	0.0313	1.9641	0.015936052
E7	0.0543	1.8268	0.029724108
G7	0.0443	1.5951	0.027772553
H1	0.0658	4.3296	0.015197709
H9	0.0575	3.3308	0.01726312
H4	0.1272	8.4288	0.015091116
B7	0.054	1.0251	0.052677788
B12	0.1094	1.5991	0.068413483
D4	0.0444	2.27	0.019559471
H1	0.0399	1.4559	0.027405728
D5	0.1019	2.4817	0.041060563
D5A2	0.8989	4.902	0.183374133

Table 1. ONPG assay with pZDisplay clones. Clones D5B7 and D5B12 had higher turnover/cell than the control D5 cells.

Western analysis of lacZ expression in clones.

Protein samples were prepared from whole cells by dissolving cells in hot SDS-PAGE loading buffer according to the method of Sambrook, et al. (Sambrook, et al., 1989). Membrane and cytosol fractions were prepared by lysing cells in hypotonic buffer and centrifuging 5 min at 14,000 g. Pellets were washed and suspended in buffer and diluted 1:1 in 2X SDS loading buffer. The supernatant (cytosol) was mixed 1:1 with 2X loading buffer. 25 μ L of each sample was run on a 14% SDS polyacrylamide gel according to the method of Laemmli (Laemmli, 1970). Proteins were transferred to nitrocellulose membrane electrophoretically and detected with mouse anti-HA antibody (Sigma Chemical Co., St. Louis, MO), 1:5,000 dilution, 1 hr incubation) and HRP-conjugated anti-mouse antibody (Sigma, 1:5,000 dilution, 1 hr incubation). Blots were developed using a bioluminescent method (100 mM Tris pH5, 0.45 mM p-coumaric acid, 70 μ g/ml luminol, and 0.01% H_2O_2) and recorded using Kodak T-Mat X-ray film (Kodak). Figure 3 below shows representative results.

Functional Assay - BSA-CUG turnover

D5 cells and stable pZDisplay transfectants D5ZB7 and D5ZB12 were plated in 96 well plates and incubated overnight with media containing either 0.5 mg/mL ortho-nitrophenyl galactopyranoside (ONPG), or 0.5 mg/mL carboxyumbelliferyl galactoside conjugated to bovine serum albumin (CUG-BSA) (100 μ L/well). ONPG is a colorless cell permeant b-galactosidase (b-Gal) substrate that is converted to orthonitrophenol (ONP), a yellow compound, upon cleavage by b-Gal. CUG-BSA is a non-fluorescent, cell-impermeant b-Gal substrate that is converted to the blue fluorescent carboxyumbelliferone-BSA conjugate (CU-BSA) upon cleavage. After overnight incubation at 23°C, the substrate-containing media was removed from the cells and measured for presence of ONP (absorbance = 405 nm) or CU-BSA (excitation = 360 nm, emission = 465 nm). The cells were then counted using the MTT assay (Plumb, et al., 1989), which produces a purple product (absorbance = 570 nm) in proportion to the number of cells present. Absorbance and fluorescence values were divided by MTT assay results to estimate b-Gal turnover/cell. In non-transfected D5 cells, there was some residual turnover of ONPG and CUG-BSA, but D5ZB7 and D5ZB12 showed significantly

higher turnover/cell values, indicating β -gal activity at the cell surface as shown in the Table below.

Cells	ONP/MTT	CUG/MTT
D5	0.197766	13505.85
D5ZB7	0.251788	22121.27
D5ZB12	0.260302	26571.68

- 5 This example demonstrates the utility of cell-surface *lacZ* β -galactosidase activity to release high-molecular weight (protein) β -galactoside conjugates from conjugation and cause extracellular activation.

EXAMPLE 36

10 CELL SURFACE EXPRESSION OF E. coli *lacY* PERMEASE IN MAMMALIAN CELLS FOR FACILITATED TRANSPORT OF GALACTOSIDE CONJUGATES

A cloning vector containing a signal sequence like the Murine Ig kappa-chain V-J2-C at the amino terminus to target proteins to the plasma membrane, and a transmembrane domain than can be fused to the carboxy terminus to anchor proteins to the membrane, was used to construct *lacY* fusions. The *lacY* gene was subcloned out of
15 frame with this transmembrane domain. The pDisplay vector (Invitrogen, Calsbad, CA) represents such a vector. The pDisplay vector map also contains an HA epitope tag that can be used to detect protein expression and the gene for neomycin resistance useful in selection with geneticin, disulfate salt (antibiotic G418).

Subcloning of *lacY* into pDisplay vector. Method A:

20 Primers designed to create appropriate restriction sites were generated to amplify *lacY* from the pcDNA3.1/His/*lacY* vector (Marker Gene Technologies). Forward primer for *lacY* (gaagatctcgggatctgtacgac) and reverse primer for *lacY* (tccccgggggcaactagaaggca) were purchased from SIGMA-Genosys (St Louis, MO). Amplification of *lacY* was performed in 3, 50 μ L reactions, with 200 ng plasmid DNA,
25 0.2 mM dNTPs, 10 mM Tris (pH 9), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 2

U Taq polymerase, and 1 μ M each of forward and reverse primers. A thermocycler was programmed for the following cycles: 1.) 94°C, 4 min. 2.) 42°C, 2 min. 3.) 72°C, 2 min. 4.) 74°C, 45 sec. 5.) 69°C, 45 sec. 6.) 72°C, 2 min. 7.) 34X steps 4 – 6. 8.) 72°C, 5 min. Reactions were run on a 0.8% agarose gel, and the major amplification product (2 kb for *lacY*) was excised from the gel and purified using a silicagel membrane like the QIAquick membrane (Qiagen, Valencia, CA) and eluted with 10 mM Tris-Cl buffer, pH 8.5.

10 10 μ g pDisplay vector and the purified *lacY* pcr product was cut with 10 Units SmaI (Roche, Indianapolis, IN) in 0.1 M Tris-acetate buffer containing 10 mM MgCl and 0.5 mM DTT, for 24 hours at 25°C. Reactions were heated to 65°C for 20 min. to inactivate SmaI. 10 Units BglII (Roche) was added to all reactions and incubated 24 hours at 37°C. Reactions were run on a 0.8% agarose gel and bands were excised and gel purified as above.

50 ng cut pDisplay vector and 37 ng *lacY* insert were incubated with 1 Unit T4 DNA ligase (Promega, Madison, WI) at 23°C for 24 hours, in a total volume of 10 μ L. Competent *E. Coli* cells were transformed with 5 μ L of ligations by heat shock method of Hanahan (Hanahan, 1983), and 100 μ L of transformations were plated on LB – ampicillin plates and incubated overnight at 37°C. Colonies from plates were inoculated into 1.4 ml terrific broth (TB) and incubated shaking at 37°C overnight. Plasmid DNA was purified from cultures using a spin miniprep and cut with BglII and SmaI enzymes as above. Plasmids that showed proper sized fragments after digestion were sequenced to confirm proper reading frame of insert. The new plasmid was named pYDisplay.

Subcloning of *lacY* into pDisplay vector. Method B:

pDisplay vector (Invitrogen, 6 μ g) was incubated with 5 units SmaI endonuclease (Roche Biochemicals) in a total reaction volume of 10 μ L with 33 mM Tris-acetate buffer (pH 7.9) containing 10 mM magnesium acetate, 66 mM potassium acetate and 0.5 mM dithiothreitol, with incubation overnight at 25°C. The pcDNA HislacY vector (Marker Gene Technologies, 5 μ g) was incubated with 5 units KpnI endonuclease (MBI Fermentas) in a total reaction volume of 10 μ L, using 10 mM Tris-HCl (pH7.5) buffer containing 10 mM MgCl₂, 0.02% Triton X-100 and 0.1mg/ml BSA; incubating 1 hr at 37°C. Reactions were quenched by ethanol precipitation and resuspended in 5 μ L Tris-EDTA buffer TE (10 mM Tris-Cl, 1 mM EDTA).

The KpnI cut pcDNAHislacY sample was again incubated with 5 units EcoRV endonuclease (Pharmacia Biotech) in a total reaction volume of 10 μ L using 20 mM Tris-acetate and 100 mM potassium acetate buffer (pH7.5) containing 20 mM magnesium acetate, incubating 1 hr at 37°C. This reaction mixture was ethanol
5 precipitated and resuspended in 9 μ L TE (10 mM Tris-Cl, 1 mM EDTA).

The cut pDisplay vector was treated with alkaline phosphatase to prevent religation of vector. 2.5 μ g cut pDisplay was incubated with 2 units alkaline phosphatase (Roche Biochemicals) in a total reaction volume of 27 μ L with 50 mM Tris-HCl, 0.1 mM EDTA (pH 8.5) for 90 minutes at 37°C. 2.5 μ L 200 mM EGTA was added to stop the
10 reaction. The reaction was heated for 10 min. at 65°C to inactivate phosphatase.

PcDNAHislacY reactions were blunted-end filled by a fill in reaction with T4 DNA polymerase (2 units) in a total reaction volume of 20 μ L with 0.1 mM dNTPs in 50 mM Tris-HCl buffer containing 15 mM $(\text{NH}_4)_2\text{SO}_4$, 7 mM MgCl_2 , 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.02 mg/ml BSA (pH 8.8), incubating at 37°C for 15
15 minutes, then stopping the reaction by incubating at 75°C for 10 min.

The above reactions were all run on a 0.75% agarose gel with the cut, phosphorylated pDisplay vector, and the 2 kb lacY insert all being excised and gel purified. Ligations were performed using T4 DNA ligase (1 unit) in reaction volumes of 10 μ L using 27 ng cut, phosphatased pDisplay vector and 33 ng lacY insert with 66 mM Tris-
20 HCl buffer containing 5 mM MgCl_2 , 1 mM dithioerythritol, and 50 μ g/mL BSA; incubating overnight at room temperature.

JM105 competent cells were transformed with the ligations by the heat shock method of Hanahan (Hanahan, 1983), and 100 μ L aliquots of transformations were plated on LB ampicillin plates. Colonies were inoculated into 1.5 ml TB broth and
25 grown with overnight shaking at 37°C. Plasmids were purified from cultures using method of Sambrook (Sambrook, et al., 1989). Plasmids were digested with Bgl II (Roche) and run on a 0.8% agarose gel. Plasmids of appropriate size were sequenced to confirm orientation and frame of insert.

Expression of pYDisplay in D5 murine tumor cells.

30 D5 cells were plated in 12 well plates and allowed to grow to 50% confluence. 1 μ g of DNA (pYDisplay or pcDNA/his/lacZ) was combined with 50 μ L

DME w/o serum. 8 μ L Lipofectamine solution (Gibco-BRL) was combined with 50 μ L DME w/o serum. DNA and lipid solutions were mixed and incubated 15 min at room temperature. Cells were rinsed with DME w/o serum and 400 μ L DME w/o serum was added to each well of cells. The DNA/lipid solutions were added to the cell media, mixed, and incubated 5 hrs at 37°C. Transfection media was then removed and replaced with RPMI + 10% calf serum. After two days growth, G418 was added to the media at a concentration of 0.7 mg/ml. Cells were maintained in selection media for two weeks and then plated in 96 well plates at a dilution of 1 cell/well. Cells were allowed to grow until colonies could be identified, then colonies were transferred to 12 well plates containing sterile 10mm cover slips.

Clones were assayed for expression of *lacY* by immunohistochemical staining. Cells were fixed in 2.5% paraformaldehyde and permeabilized in microtubule stabilizing buffer with 0.5% Triton X-100. Cells were blocked in PBS with 0.3% BSA and incubated with mouse anti-HA antibody (SIGMA) diluted 1:100 in PBS with 0.3% BSA for 1 hr at room temperature, then washed in PBS and incubated with FITC conjugated anti-mouse antibody (SIGMA) for 2 hrs at room temperature. The D5/pYDisplay-A2 clone showed good staining.

Functional Assay: Internalization of FITC labeled asialofetuin fragments.

Asialofetuin (Sigma) was labeled with fluorescein isothiocyanate (FITC) as follows. A solution of FITC (423 μ L of a 5 mg/mL in 95% ethanol) was added to a solution of asialofetuin (25 mg) in 0.2 M NaHCO_3 - Na_2CO_3 buffer (pH 9) (6.25 mL). After stirring for 1.5 hrs. the reaction mixture was dialyzed, and the absorbance at 280 nm and 493 nm measured (Shimadzu UV-1201 spectrometer), indicating a labeling of approximately 11 moles fluorescein / mole asialofetuin.

The FITC-labeled asialofetuin (FITC-AF, 2 mg) was incubated with α -chymotrypsin on beaded agarose (4.5 mg, 0.56 U/mg, Sigma) at 30°C for 4 hours, shaken periodically. FITC-labeled asialofetuin (FITC-AF, 2 mg) was also incubated with elastase (10 μ L of a 6.5 mg/mL solution, 5U/mg) in sodium phosphate buffer (pH 8) for 18 hours at 25°C. 10 μ L of each reaction was combined with 2X tricine sample buffer (10 μ L) and incubated at 40°C for 60 min. The sample was then run on a 16% acrylamide, 1% bis gel (4% stacker) with Tris/Tricine running buffer and 3 μ L of ultra-low prestained

markers (Sigma) at 30V until samples ran through the stacker, and then at 100 V until coomassie blue stain ran off the gel. The gel was stained with coomassie blue for 1 hour, and then destained in 10% acetic acid. The gel indicated approximately 12 enzyme fragments were produced, with molecular weights from 1-20K, all of about equal concentration.

D5 and stable pYDisplay transfectant D5/pYDisplay-A2 cells were plated in 96 well plates at a density of 2×10^5 cells/mL and incubated overnight with media containing either 0.5 mg/mL FITC-labeled asialofetuin (FITC-AF), or 0.5 mg/mL FITC-labeled asialofetuin that had been digested with (FITC-AF fragments) (100 μ L/well).

After staining for 2 hours, the cells were removed from the media, washed with PBS (500 μ L) and fixed using ice-cold methanol (0.5 mL) for 10 min. Cover slips were mounted and fluorescence microscopic analysis (Nikon Labophot-2, Nikon, Inc., Melville, NY) indicated green fluorescent staining of pYDisplay transfectant D5/pYDisplay-A2 cells with the FITC-AF fragments solution versus control D5 cells using excitation at 485 nm.

This example demonstrates the utility of cell-surface *E. coli lacY* expression to permeabilize high-molecular weight b-galactoside conjugates.

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CLAIMS

What is claimed is:

- 1 1. A method of targeted delivery of a bioactive compound comprising:
2 (a) introducing into targeted cells a gene encoding an enzyme;
3 (b) administering an inactive conjugate of the bioactive compound;
4 wherein the enzyme releases the bioactive compound from
5 conjugation.
- 1 2. The method of claim 1 wherein the enzyme does not naturally occur in the
2 targeted cells in an amount effective to release the bioactive compound from conjugation
- 1 3. The method of claim 1 wherein the enzyme is an hydrolitic enzyme.
- 1 4. The method of claim1 wherein the enzyme is a marker gene.
- 1 5. The method of claim 1 wherein the enzyme is an active component of *lacZ*
2 β -galactosidase from the bacteria *Escherichia coli*.
- 1 6. The method of claim 1 wherein the enzyme is an active component of *GUS*
2 β -glucuronidase from the bacteria *Escherichia coli*.
- 1 7. The method of claim 1 wherein the enzyme is an active component of
2 firefly luciferase from *Photinus pyralis*.
- 1 8. The method of claim 1 wherein the enzyme is an active component of
2 firefly luciferase from *Renilla reniformis*.
- 1 9. The method of claim 1 wherein the enzyme is an active component of β -
2 lactamase from the bacteria *Escherichia coli*.

1 10. The method of claim 1 wherein the enzyme is an active component of
2 alkaline phosphatase.

1 11. The method of claim 1 wherein the bioactive compound is selected from
2 the group consisting of cycloheximide (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-O-(β -D-
3 galactopyranosyl)ethyl]glutarimide), dexamethasone, 4'-Hydroxymethyl-
4 trimethylpsoralen, chloramphenicol, 5-fluorouridine, tetracycline, doxorubicin, resveratrol,
5 phorbol octanoate acetate, dioctanoyl glycerol, 4-hydroxyphenylretinamide, mitoxantrone,
6 and thymidine.

1 12. A method of hindering cell growth comprising:
2 (a) introducing into targeted cells a gene encoding an enzyme; and
3 (b) administering in an amount effect to hinder growth of the targeted
4 cells an inactive conjugate of a bioactive compound;
5 wherein the enzyme releases the bioactive compound from
6 conjugation.

1 13. The method of claim 12 wherein the targeted cells are the cancer cells.

1 14. The method of claim 13 wherein the targeted cells are proximal to the
2 cancer cells.

1 15. The method of claim 13 wherein the drug initiates a host immune response
2 to the cancer cells.

1 16. The method of claim 13 wherein the cancer cells are present in a mammal.

1 17. The method of claim 12 wherein the cells are plant cells.

- 1 18. The method of claim 12 where in the cells are bacterial cells.
- 1 19. The method of claim 12 wherein the enzyme does not naturally occur in the
2 targeted cells in an amount effective to release the bioactive compound from conjugation.
- 1 20. The method of claim 12 wherein the enzyme is an hydrolitic enzyme.
- 1 21. The method of claim 12 wherein the enzyme is a marker gene.
- 1 22. The method of claim 12 wherein the enzyme is an active component of
2 *lacZ* β -galactosidase from the bacteria *Escherichia coli*.
- 1 23. The method of claim 12 wherein the enzyme is an active component of
2 *GUS* β -glucuronidase from the bacteria *Escherichia coli*.
- 1 24. The method of claim 12 wherein the enzyme is an active component of
2 firefly luciferase from *Photinus pyralis*.
- 1 25. The method of claim 12 wherein the enzyme is an active component of
2 firefly luciferase from *Renilla reniformis*.
- 1 26. The method of claim 12 wherein the enzyme is an active component of β -
2 lactamase from the bacteria *Escherichia coli*.
- 1 27. The method of claim 12 wherein the enzyme is an active component of
2 alkaline phosphatase.
- 1 28. A method of enhancing cell growth comprising:
2 (a) introducing into the cells a gene encoding an enzyme; and

3 (b) administering a conjugate of a bioactive compound in an amount
4 effect to enhance cell growth an inactive conjugate of a bioactive compound;
5 wherein the bioactive compound enhances cell growth and the
6 enzyme releases the bioactive compound from conjugation.

1 29. The method of claim 28 wherein enhancing cell growth increases the
2 production of a protein in a cell culture.

1 30. The method of claim 28 wherein the enzyme does not naturally occur in the
2 targeted cells in an amount effective to release the bioactive compound from conjugation.

1 31. The method of claim 28 wherein the enzyme is an hydrolitic enzyme.

1 32. The method of claim 28 wherein the enzyme is a marker gene.

1 33. The method of claim 28 wherein the enzyme is an active component of
2 *lacZ* β -galactosidase from the bacteria *Escherichia coli*.

1 34. The method of claim 28 wherein the enzyme is an active component of
2 *GUS* β -glucuronidase from the bacteria *Escherichia coli*.

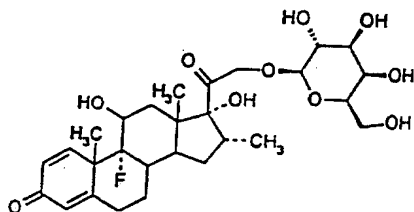
1 35. The method of claim 28 wherein the enzyme is an active component of
2 firefly luciferase from *Photinus pyralis*.

1 36. The method of claim 28 wherein the enzyme is an active component of
2 firefly luciferase from *Renilla reniformis*.

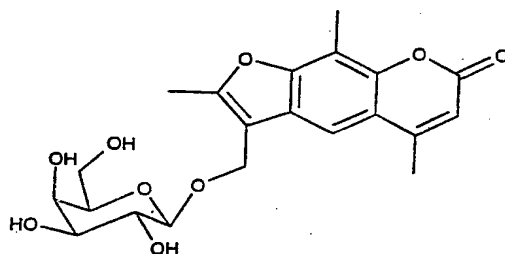
1 37. The method of claim 28 wherein the enzyme is an active component of β -
2 lactamase from the bacteria *Escherichia coli*.

- 1 38. The method of claim 28 wherein the enzyme is an active component of
2 alkaline phosphatase.

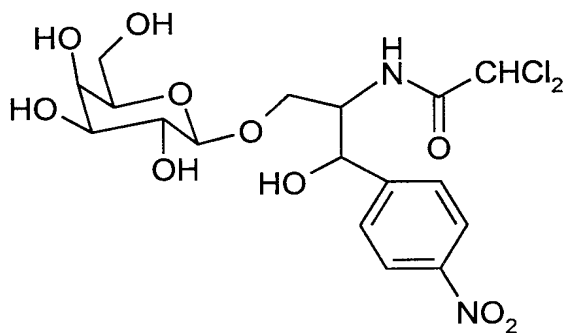
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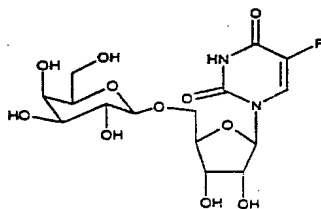
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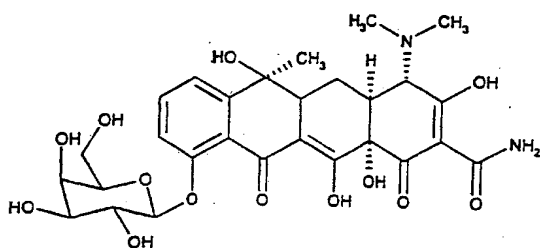
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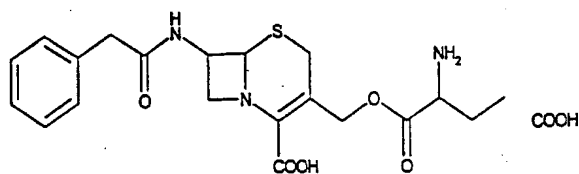
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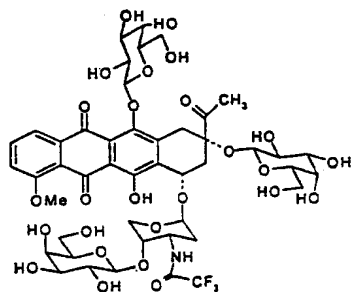
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44. An inactive conjugate of a bioactive compound having the formula

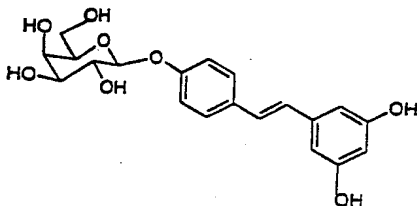


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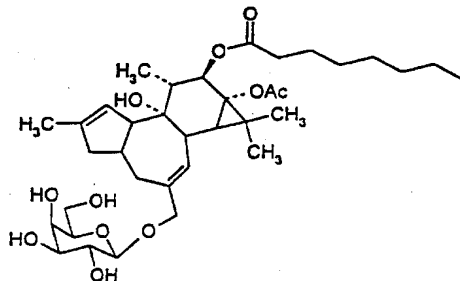


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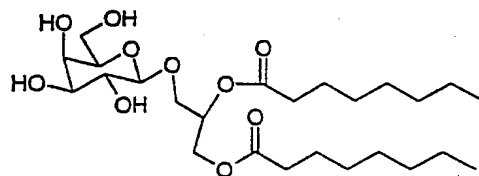
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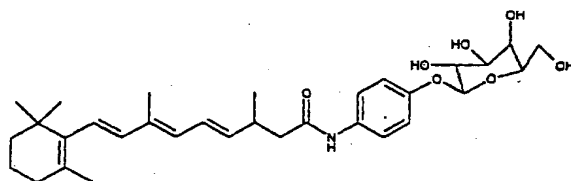
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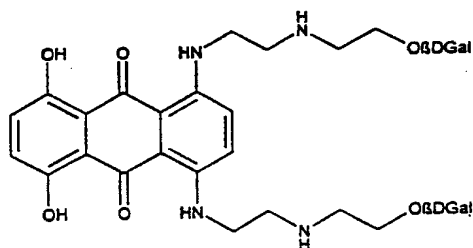
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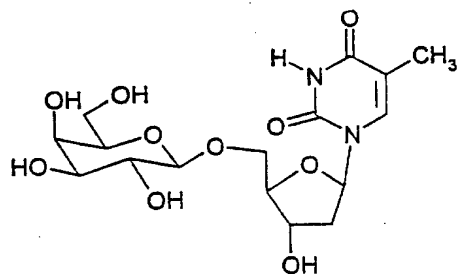
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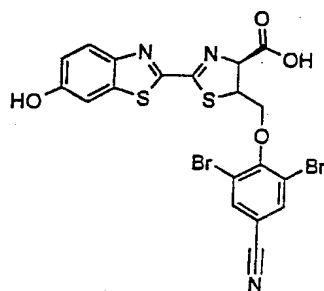
50. An inactive conjugate of a bioactive compound having the formula



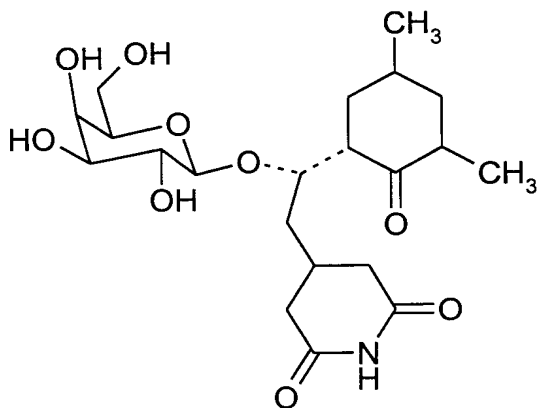
51. An inactive conjugate of a bioactive compound having the formula



52. An inactive conjugate of a bioactive compound having the formula



53. An inactive conjugate of a bioactive compound having the formula



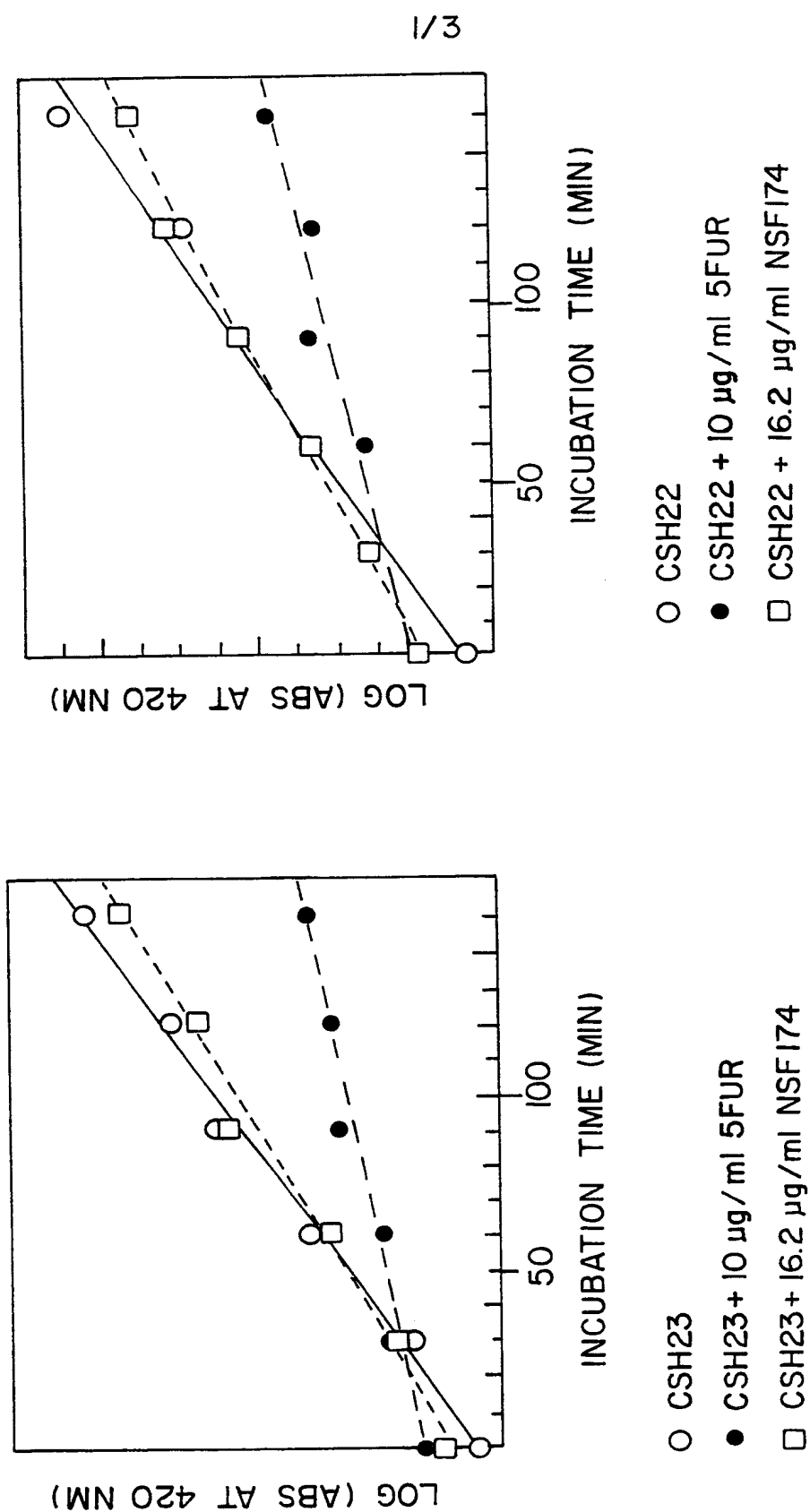
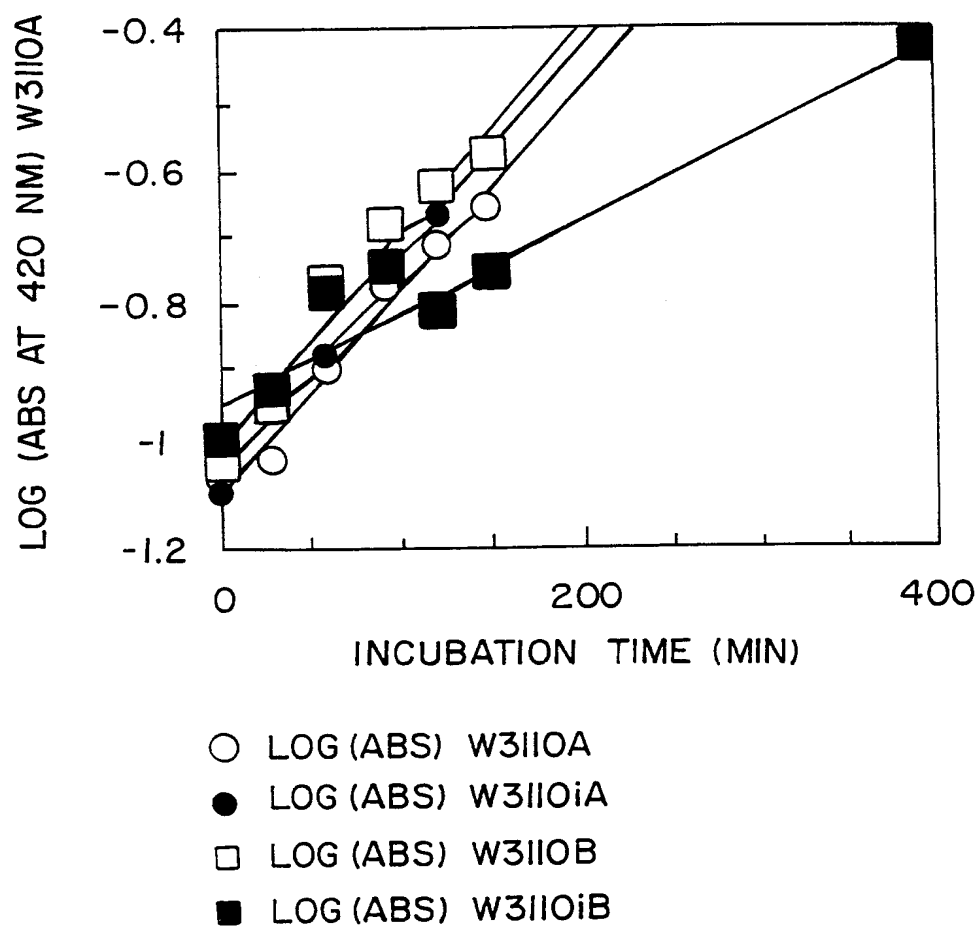


FIG. 1.

2/3

**FIG. 2.**

A

D5 + pYDisplay A2

D5A2

M**W****C****M****W****C****B**

D5 + pZDisplay B7

D5 + pZDisplay B12

D5A2

S**M****W****C****M****W****C****M****C**

FIG. 3.

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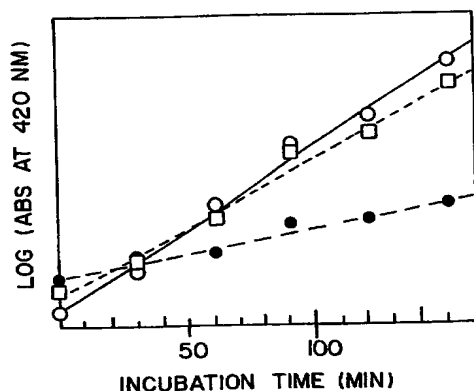
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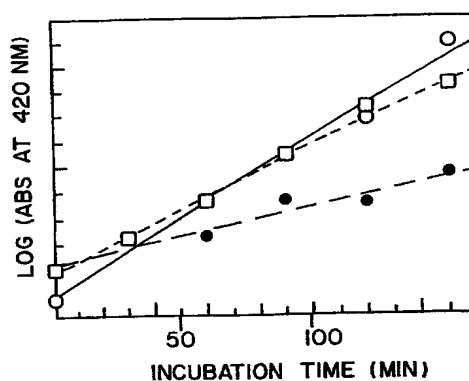
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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR TARGETED ENZYMATIC RELEASE OF CELL REGULATORY COMPOUNDS



- CSH23
- CSH23 + 10 µg/ml 5FUR
- CSH23 + 16.2 µg/ml NSF174



- CSH22
- CSH22 + 10 µg/ml 5FUR
- CSH22 + 16.2 µg/ml NSF174

(57) Abstract: Novel pro-drugs and methods for their use to alter the growth and biological characteristics of living cells, tissues, or whole organisms are described. The methods allow for selective activation of the pro-drugs at or near transformant host cells expressing a gene for an enzyme that activates the pro-drugs. Pro-drugs according to a preferred embodiment of the invention are conjugates of a bioactive compound and a chemical group that is capable of being cleaved from the bioactive compound by action of an enzyme. Methods according to this invention include: (a) introducing into targeted cells a gene encoding an enzyme and (b) administering a pro-drug, wherein the enzyme releases the pro-drug from conjugation. In a preferred embodiment of the invention, the gene encoding the enzyme is a marker gene.

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INTERNATIONAL SEARCH REPORT

In ternational Application No

PCT/US 00/15156

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K48/00 C12N9/38 C12N9/02 C12N9/86 C07H15/24
C07H15/26 C07H15/203 C07H15/252 C07H15/244 C07H19/073

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE CANCERLIT 'Online! US NATIONAL CANCER INSTITUTE D. FARQUHAR ET AL.,: "Antitumor galactoside prodrugs (Meeting abstract)" XP002153500 Database Accession number (NCI) 98641121; (Dialog) 01317043 * abstract * & Proc. Annu. Meet Am. Assoc. Cancer Research 1997, Vol. 38, page A4121 --- -/--</p>	<p>1-5, 11-13, 16, 19-22</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* & * document member of the same patent family

Date of the actual completion of the international search

23 November 2000

Date of mailing of the international search report

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Name and mailing address of the ISA

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Authorized officer

Julia, P

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/15156

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US D. FARQUHAR ET AL.,: "Antitumor galactoside prodrugs" XP002153501 Database Accession number PREV199799533520 * abstract * & Proc. Annu. Meet. Am. Assoc. Cancer Research Annual Meeting 1997, Vol. 38, pages 613-614</p>	<p>1-5, 11-13, 16,19-22</p>
X	<p>----- DATABASE CANCERLIT 'Online! US NATIONAL CANCER INSTITUTE F. MARINI ET AL.,: "E. coli LacZ as a suicide gene for anticancer prodrug activation (Meeting abstract)" XP002153502 Database Accession number (NCI) 98639294; (Dialog) 01315220 * abstract * & Proc. Annu. Meet. Am. Assoc. Cancer Res 1997, Vol. 38, page A2294</p>	<p>1-5, 11-13, 16,19-22</p>
X	<p>----- DATABASE PUBMED 'Online! NATIONAL LIBRARY OF MEDICINE (NCBI) WA DENNY ET AL.,: "The design of selectively-activated anti-cancer prodrugs for use in antibody-directed and gene-directed enzyme-prodrug therapies" XP002153503 Database Accession number PMID 9625483 * abstract * & J. Pharm. Pharmacol. 1998, Vol. 50 (4), pages 387-394</p>	<p>1,2, 12-14, 16,19</p>
X	<p>----- WO 98 30709 A (CHADA SUNIL ;JOLLY DOUGLAS J (US); MOORE MARGARET DOW (US)) 16 July 1998 (1998-07-16)</p>	<p>1-4, 11-16, 19-21</p>
Y	<p>* the whole document, in particular page 24 lines 13-29, examples 2-3 and figure 5 *</p>	<p>28-33</p>
Y	<p>----- WO 96 15435 A (IDEXX LAB INC) 23 May 1996 (1996-05-23) the whole document -----</p>	<p>28-33</p>
	<p>----- -/--</p>	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/15156

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US E. BAKINA ET AL.,: "Intensely cytotoxic anthracycline prodrugs : galactosides." XP002153504 Database Accession number PREV200000310617 * abstract * & Anti-Cancer Drug Design 1999 (Dec), Vol. 14 (6), pages 507-515 -----</p>	<p>1-5, 11-13, 16,19-22</p>

INTERNATIONAL SEARCH REPORT

...ternational application No.
PCT/US 00/15156

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-38 (as far as in vivo) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4, 11-21, 28-32(partial), 5, 22, 33 (complete)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-4, 11-21, 28-32 (partial); 5, 22,33 (complete)

a method of targeted delivery of a bioactive compound comprising (a) introducing into targeted cells a gene encoding an enzyme (b) administering an inactive conjugate of the bioactive compound, wherein the enzyme releases the bioactive compound from conjugation, the enzyme does not naturally occur in the targeted cells in an amount effective to release the bioactive compound from conjugation, the enzyme is an hydrolitic marker enzyme and it is an active component of lacZ beta-galactosidase from the bacteria Escherichia coli. Said method of targeted delivery wherein the bioactive compound hinders growth of the targeted cells and wherein the targeted cells are cancer cells (or proximal to these cells), plant cells or bacterial cells and wherein said cancer cells are present in a mammal. Said method wherein the bioactive compound initiates a host immune response to the cancer cells. Said method of targeted delivery wherein the bioactive compound enhances the growth of the targeted cells, wherein said enhancing cell growth increases the production of a protein in a cell culture.

2. Claims: 1-4, 11-21, 28-32 (partial); 6, 23,34 (complete)

a method of targeted delivery of a bioactive compound comprising (a) introducing into targeted cells a gene encoding an enzyme (b) administering an inactive conjugate of the bioactive compound, wherein the enzyme releases the bioactive compound from conjugation, the enzyme does not naturally occur in the targeted cells in an amount effective to release the bioactive compound from conjugation, the enzyme is an hydrolitic marker enzyme and it is an active component of GUS beta-glucuronidase from the bacteria Escherichia coli. Said method of targeted delivery wherein the bioactive compound hinders growth of the targeted cells and wherein the targeted cells are cancer cells (or proximal to these cells), plant cells or bacterial cells and wherein said cancer cells are present in a mammal. Said method wherein the bioactive compound initiates a host immune response to the cancer cells. Said method of targeted delivery wherein the bioactive compound enhances the growth of the targeted cells, wherein said enhancing cell growth increases the production of a protein in a cell culture.

3. Claims: 1-4, 11-21, 28-32 (partial); 7, 24, 35 (complete)

a method of targeted delivery of a bioactive compound comprising (a) introducing into targeted cells a gene encoding an enzyme (b) administering an inactive conjugate of the bioactive compound, wherein the enzyme releases the bioactive compound from conjugation, the enzyme does not naturally occur in the targeted cells in an amount effective to release the bioactive compound from conjugation, the

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

enzyme is an hydrolitic marker enzyme and it is an active component of firefly luciferase from *Photinus pyralis* or from *Renilla reniformis*. Said method of targeted delivery wherein the bioactive compound hinders growth of the targeted cells and wherein the targeted cells are cancer cells (or proximal to these cells), plant cells or bacterial cells and wherein said cancer cells are present in a mammal. Said method wherein the bioactive compound initiates a host immune response to the cancer cells. Said method of targeted delivery wherein the bioactive compound enhances the growth of the targeted cells, wherein said enhancing cell growth increases the production of a protein in a cell culture.

4. Claims: 1-4, 11-21, 28-32 (partial); 9, 26, 37 (complete)

a method of targeted delivery of a bioactive compound comprising (a) introducing into targeted cells a gene encoding an enzyme (b) administering an inactive conjugate of the bioactive compound, wherein the enzyme releases the bioactive compound from conjugation, the enzyme does not naturally occur in the targeted cells in an amount effective to release the bioactive compound from conjugation, the enzyme is an hydrolitic marker enzyme and it is an active component of beta-lactamase from the bacteria *Escherichia coli*. Said method of targeted delivery wherein the bioactive compound hinders growth of the targeted cells and wherein the targeted cells are cancer cells (or proximal to these cells), plant cells or bacterial cells and wherein said cancer cells are present in a mammal. Said method wherein the bioactive compound initiates a host immune response to the cancer cells. Said method of targeted delivery wherein the bioactive compound enhances the growth of the targeted cells, wherein said enhancing cell growth increases the production of a protein in a cell culture.

5. Claims: 1-4, 11-21, 28-32 (partial); 10, 27, 38 (complete)

a method of targeted delivery of a bioactive compound comprising (a) introducing into targeted cells a gene encoding an enzyme (b) administering an inactive conjugate of the bioactive compound, wherein the enzyme releases the bioactive compound from conjugation, the enzyme does not naturally occur in the targeted cells in an amount effective to release the bioactive compound from conjugation, the enzyme is an hydrolitic marker enzyme and it is an active component of alkaline phosphatase. Said method of targeted delivery wherein the bioactive compound hinders growth of the targeted cells and wherein the targeted cells are cancer cells (or proximal to these cells), plant cells or bacterial cells and wherein said cancer cells are present in a mammal. Said method wherein the bioactive compound initiates a host immune response to the cancer cells. Said method of targeted delivery wherein the bioactive compound enhances the growth

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

of the targeted cells, wherein said enhancing cell growth increases the production of a protein in a cell culture.

6. Claim : 39 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a dexamethasone galactoside conjugate (example 2)

7. Claim : 40 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a hydroxymethylpsoralen galactoside conjugate (example 3)

8. Claim : 41 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a chloramphenicol galactoside conjugate (example 15)

9. Claim : 42 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a 5-fluorouridine-5'-O-beta-D-galactoside conjugate (example 16)

10. Claim : 43 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a tetracycline galactoside conjugate (example 17)

11. Claim : 44 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a benzyl cephalothin L-glutamate conjugate (example 18)

12. Claim : 45 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a doxorubicin galactoside conjugate (example 19)

13. Claim : 46 (complete)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a resveratrol galactoside conjugate (example 20)

14. Claim : 47 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a phorbol octanoate acetate galactoside conjugate (example 21)

15. Claim : 48 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a dioctanoyl glycerol galactoside conjugate (example 22)

16. Claim : 49 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a 4-O-galactosyl phenyl retinamide conjugate (example 23)

17. Claim : 50 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a mitoxantrone di-galactoside conjugate (example 24)

18. Claim : 51 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a thymidine 5-O-beta-D-galactoside conjugate (example 25)

19. Claim : 52 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a D-luciferin-bromoxynil conjugate (example 27)

20. Claim : 53 (complete)

an inactive conjugate of a bioactive compound, wherein said inactive conjugate is a cycloheximide galactoside conjugate (example 1)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/15156

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9830709 A	16-07-1998	AU 5918098 A EP 0970232 A	03-08-1998 12-01-2000
WO 9615435 A	23-05-1996	US 5620865 A BR 9505075 A EP 0871854 A	15-04-1997 21-10-1997 21-10-1998

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
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(10) International Publication Number
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C12N 9/38, 9/02, 9/86, C07H 15/24, 15/26, 15/203,
15/252, 15/244, 19/073

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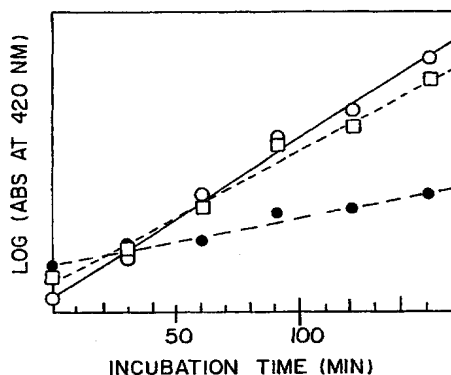
(26) Publication Language: English

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Filed on 30 June 1999 (30.06.1999)(71) Applicant (for all designated States except US):
MARKER GENE TECHNOLOGIES, INC. [US/US];
1850 Millrace Drive, Eugene, OR 97403-1927 (US).

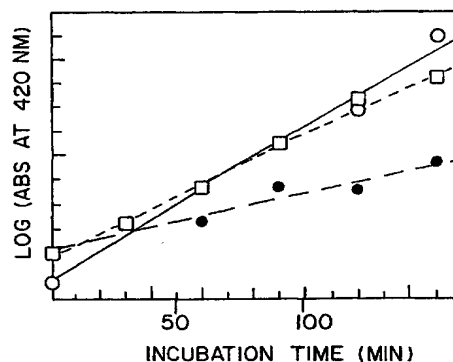
(72) Inventors; and

(75) Inventors/Applicants (for US only): **NALEWAY, John**
[US/US]; 3247 Lincoln Street, Eugene, OR 97405-2773
(US). **HOWARD, Rachel** [US/US]; 1333 Oakpatch Road,
No. 17, Eugene, OR 97402 (US).(74) Agent: **SHUSTER, Michael**; McCutchen, Doyle, Brown
& Enersen, LLP, Three Embarcadero Center, San Fran-
cisco, CA 94111 (US).(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE,
DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU,
ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,
LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO,
NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR,
TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR TARGETED ENZYMATIC RELEASE OF CELL REGULATORY COM-
POUNDS

○ CSH23
● CSH23 + 10 µg/ml 5FUR
□ CSH23 + 16.2 µg/ml NSF174



○ CSH22
● CSH22 + 10 µg/ml 5FUR
□ CSH22 + 16.2 µg/ml NSF174

(57) Abstract: Novel pro-drugs and methods for their use to alter the growth and biological characteristics of living cells, tissues, or whole organisms are described. The methods allow for selective activation of the pro-drugs at or near transformant host cells expressing a gene for an enzyme that activates the pro-drugs. Pro-drugs according to a preferred embodiment of the invention are conjugates of a bioactive compound and a chemical group that is capable of being cleaved from the bioactive compound by action of an enzyme. Methods according to this invention include: (a) introducing into targeted cells a gene encoding an enzyme and (b) administering a pro-drug, wherein the enzyme releases the pro-drug from conjugation. In a preferred embodiment of the invention, the gene encoding the enzyme is a marker gene.



Published:

- with international search report
- with amended claims

Date of publication of the amended claims: 23 August 2001

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19 July 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

AMENDED CLAIMS

[received by the International Bureau on 9 April 2001 (09.04.01);
original claims 1, 11, 12, and 28 amended; original claims 2, 19, 30 and 42 cancelled;
remaining claims unchanged (6 pages)]

- 1 1. A method of targeted delivery of a bioactive compound comprising:
2 (a) introducing into targeted cells a gene encoding an enzyme, wherein
3 said enzyme does not naturally occur in the targeted cells in an amount effective to release
4 the bioactive compound from conjugation;
5 (b) administering an inactive conjugate of the bioactive compound, said
6 inactive conjugate having structure BLOCK-X-DRUG;
7 wherein the enzyme releases the bioactive compound from
8 conjugation, said released bioactive compound having structure H-X-DRUG.
- 1 3. The method of claim 1 wherein the enzyme is an hydrolitic enzyme.
- 1 4. The method of claim 1 wherein the enzyme is a marker gene.
- 1 5. The method of claim 1 wherein the enzyme is an active component of *lacZ*
2 β -galactosidase from the bacteria *Escherichia coli*.
- 1 6. The method of claim 1 wherein the enzyme is an active component of *GUS*
2 β -glucuronidase from the bacteria *Escherichia coli*.
- 1 7. The method of claim 1 wherein the enzyme is an active component of
2 firefly luciferase from *Photinus pyralis*.
- 1 8. The method of claim 1 wherein the enzyme is an active component of
2 firefly luciferase from *Renilla reniformis*.
- 1 9. The method of claim 1 wherein the enzyme is an active component of β -
2 lactamase from the bacteria *Escherichia coli*.

1 10. The method of claim 1 wherein the enzyme is an active component of
2 alkaline phosphatase.

1 11. The method of claim 1 wherein the bioactive compound is selected from
2 the group consisting of cycloheximide, dexamethasone, 4'-hydroxymethyl-
3 trimethylpsoralen, chloramphenicol, 5-fluorouridine, tetracycline, doxorubicin,
4 resveratrol, phorbol octanoate acetate, dioctanoyl glycerol, 4-hydroxyphenylretinamide,
5 mitoxantrone, and thymidine.

1 12. A method of hindering cell growth comprising:
2 (a) introducing into targeted cells a gene encoding an enzyme; and
3 (b) administering in an amount effective to hinder growth of the targeted
4 cells an inactive conjugate of a bioactive compound, said inactive conjugate having
5 structure BLOCK-X-DRUG;
6 wherein the enzyme does not naturally occur in the targeted cells in
7 an amount effective to release the bioactive compound from conjugation, and the enzyme
8 releases the bioactive compound from conjugation, said released bioactive compound
9 having structure H-X-DRUG.

1 13. The method of claim 12 wherein the targeted cells are the cancer cells.

1 14. The method of claim 13 wherein the targeted cells are proximal to the
2 cancer cells.

1 15. The method of claim 13 wherein the drug initiates a host immune response
2 to the cancer cells.

1 16. The method of claim 13 wherein the cancer cells are present in a mammal.

1 17. The method of claim 12 wherein the cells are plant cells.
1

- 1 18. The method of claim 12 where in the cells are bacterial cells.
- 1 20. The method of claim 12 wherein the enzyme is an hydrolitic enzyme.
- 1 21. The method of claim 12 wherein the enzyme is a marker gene.
- 1 22. The method of claim 12 wherein the enzyme is an active component of
2 *lacZ* β -galactosidase from the bacteria *Escherichia coli*.
- 1 23. The method of claim 12 wherein the enzyme is an active component of
2 *GUS* β -glucuronidase from the bacteria *Escherichia coli*.
- 1 24. The method of claim 12 wherein the enzyme is an active component of
2 firefly luciferase from *Photinus pyralis*.
- 1 25. The method of claim 12 wherein the enzyme is an active component of
2 firefly luciferase from *Renilla reniformis*.
- 1 26. The method of claim 12 wherein the enzyme is an active component of β -
2 lactamase from the bacteria *Escherichia coli*.
- 1 27. The method of claim 12 wherein the enzyme is an active component of
2 alkaline phosphatase.
- 1 28. A method of enhancing cell growth comprising:
2 (a) introducing into the cells a gene encoding an enzyme; and
3 (b) administering a conjugate of a bioactive compound in an amount
4 effective to enhance cell growth an inactive conjugate of a bioactive compound having
5 structure BLOCK-X-DRUG;

6 wherein the bioactive compound has structure H-X-DRUG, and
7 enhances cell growth and the enzyme does not naturally occur in the cells in an amount
8 effective to release the bioactive compound from conjugation, and the enzyme releases the
9 bioactive compound from conjugation.

1 29. The method of claim 28 wherein enhancing cell growth increases the
2 production of a protein in a cell culture.

1 31. The method of claim 28 wherein the enzyme is an hydrolitic enzyme.

1 32. The method of claim 28 wherein the enzyme is a marker gene.

1 33. The method of claim 28 wherein the enzyme is an active component of
2 *lacZ* β -galactosidase from the bacteria *Escherichia coli*.

1 34. The method of claim 28 wherein the enzyme is an active component of
2 *GUS* β -glucuronidase from the bacteria *Escherichia coli*.

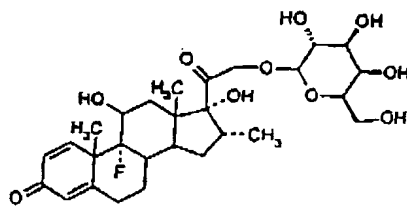
1 35. The method of claim 28 wherein the enzyme is an active component of
2 firefly luciferase from *Photinus pyralis*.

1 36. The method of claim 28 wherein the enzyme is an active component of
2 firefly luciferase from *Renilla reniformis*.

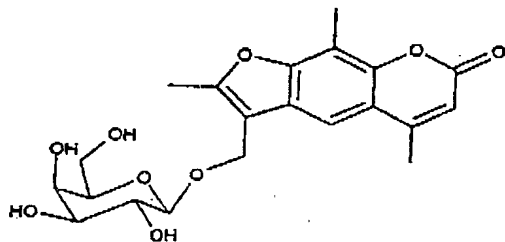
1 37. The method of claim 28 wherein the enzyme is an active component of β -
2 lactamase from the bacteria *Escherichia coli*.

1 38. The method of claim 28 wherein the enzyme is an active component of
2 alkaline phosphatase.

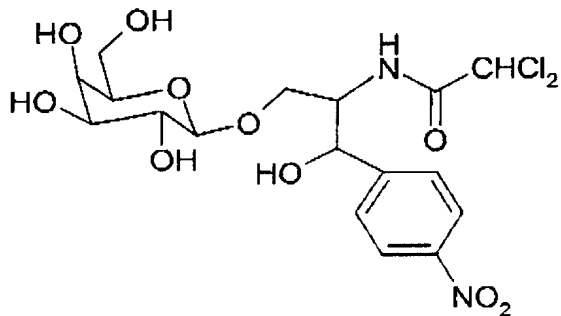
39. An inactive conjugate of a bioactive compound having the formula



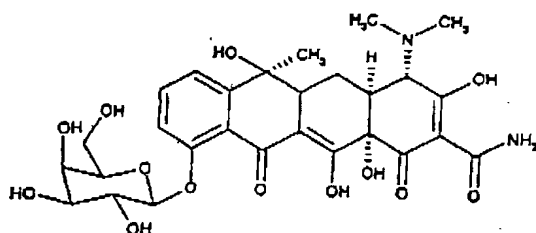
40. An inactive conjugate of a bioactive compound having the formula



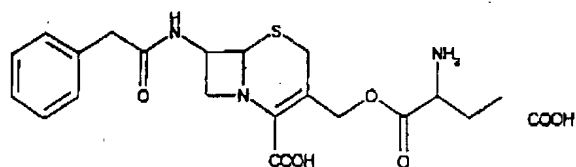
41. An inactive conjugate of a bioactive compound having the formula



43. An inactive conjugate of a bioactive compound having the formula



44. An inactive conjugate of a bioactive compound having the formula



45. An inactive conjugate of a bioactive compound having the formula

